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14. ABSTRACT Because of the potential synergistic interaction between an anti-angiogenic aminosterol, squalamine, and other angiogenic modifiers such as vascular endothelial growth factor (VEGF) and cytokines that may be released during intermittent androgen withdrawal therapy, we tested extensively the interaction between squalamine and VEGF for an enhanced cytotoxicity to human prostate cancer cells in vitro and xenografts tumor models in vivo. While in vitro synergistic interaction was demonstrated specifically in human prostate cancer cell lines containing a functional androgen receptor, we encountered difficulty in demonstrating such synergism in vivo for the reason that severe toxicity was noted when VEGF was delivered as an Ad-CMV-TK vector. For this reason, we explored other possible synergistic interaction between squalamine and castration. Squalamine is highly synergistic to castration-induced endothelial destruction when applied at the time of castration. We noted VEGF receptor, flt-1 and integrin profile (e.g. α6β4) can predict squalamine response. Prostate cancer cells lacking the expression of these markers may be less responsive to the synergistic interaction between squalamine and castration. We are currently assessing the possible interaction between squalamine and VEGF and squalamine and androgen status of the cell culture and in animals subjected to castration to evaluate if synergism may exist particularly against the growth of endothelial cells.					
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## Introduction:

The objective of this proposal is to seek for a combination therapy between a low molecular weight aminosterol squalamine which has anti-angiogenic activity against induced endothelial proliferation and migration and vascular endothelial growth factor, VEGF, on the growth of human prostate tumors both *in vitro* and *in vivo*. Although both agents when applied alone have little anti-tumor effect, they have remarkable synergistic action when applied together in tumor cells that express certain profiles of integrin isotypes and VEGF receptors. This approach is taken because of the known inherent genetic stability of endothelial cells which are required for tumor cells' continued growth and expansion and the potential clinical application of an effective combination therapy targeted at tumor and its endothelial supplies for the effective treatment of hormone refractory prostate cancers.

## Body:

### Task 1: Establishment of *in vivo* human prostate tumors:

This task has been completed. Please see previous progress report.

### Task 2: Construction, characterization and production of adenoviruses that contain VEGF driven by a CMV universal promoter:

This task has been completed. Please see previous progress report.

### Task 3: Evaluation of the *in vitro* and *in vivo* synergism between squalamine and VEGF (or castration), and assessment of the biochemical and morphologic changes of the prostatic tissues *in vivo*:

This task has been completed. Please see previous progress report.

### Task 4: Determine the *in vitro* effect of squalamine and/or VEGF on the growth of prostatic and endothelial cells:

This task has been completed and published (Jin et al., Cancer Gene Therapy 12:257-267, 2005).

### Task 5: Recording of the morphologic changes of cells after squalamine and/or VEGF treatment:

This task has been completed. Please see previous progress report.

### Task 6: Evaluation of the relationship between morphologic changes of prostate cancer and endothelial cells *in vitro* after squalamine and/or VEGF treatment with that of their biochemical expression of TSP-1 and cell surface integrin isotypes:

In a previous progress report, we have completed the analysis of morphologic and biochemical features of prostate tumors after squalamine treatment in intact and castrated hosts. We are evaluating TSP-1 gene expression in prostate cancer cell lines with different antibodies. We have not found a good antibody that can provide reproducible assessment of the expression of this antigen in prostate cancer cell lines. However, we have focused our study on  $\alpha_v\beta_5$  integrin expression in prostate cancer cells and have completed a study to show that an anti-PAN  $\alpha_v$  antibody can abrogate the ability of prostate cancer cells to attach, migrate, and invade through vitronectin substratum. The interruption of this interaction resulted in marked enhancement of apoptosis. These data are provided as Appendix 1.

**Task 7:** Confirmation of the above biochemical responses of prostate cancer cells and endothelial cells to squalamine and VEGF *in vivo*.

We have completed the evaluation of HUVEC cell culture *in vitro* to the effect of squalamine. We have reported a part of these findings in *Cancer Gene Therapy* (Vol. 12: 257-267, 2005).

**Task 8:** Evaluation of methodologies for evaluating signal cascade and apoptosis following VEGF and squalamine.

This task has been completed. We are in the process of preparing a manuscript indicating the effect of VEGF and squalamine on phosphorylation of PP125FAK and Pyk2.

**Task 9:** Evaluation of changes in signal transduction components following exposure to squalamine and/or VEGF *in vitro* and confirmation of such changes in prostate tumor models *in vivo*.

We have evaluated soluble paracrine mediators that may be responsible for the induction of VEGF in prostate cancer and prostate or bone stroma cells *in vitro*. We discovered that a soluble protein factor called beta-2 microglobulin is responsible for the induction of VEGF and its receptor neuropilin-1 to form an autocrine loop supporting the growth of cancer cells. These results open up a new avenue of investigation, which could impact our understanding of the role of squalamine and VEGF loop in prostate cancer cells.

**Task 10:** Characterization of changes of signal transduction components and their relationship to apoptosis, and comparison of their activity both *in vivo* and *in vitro*.

See above.

### **Key Research Accomplishments:**

- We have completed a manuscript indicating co-targeting tumor and tumor-associated endothelium using gene therapy as a prototype resulted in improved affect in eradicating cancer growth as xenografts in culture. This same study also has been confirmed *in vitro* (Jin et al., *Cancer Gene Therapy* 12:257-267, 2005).
- We are establishing the basic methodology to study signal transduction in prostate cancer cells and the same methodologies will be used to measure signaling pathway in response to squalamine and/or VEGF. We have established a new model system to study the inter-relationship between beta-2 microglobulin, VEGF, and VEGF receptor signaling in prostate cancer cells.

### **Reportable Outcomes:**

1. A manuscript by Jin et al. was published in *Cancer Gene Therapy*.
2. A review article by Chung et al. was published in *Journal of Urology*.
3. A new grant was submitted to NIH based upon the VEGF receptor and soluble factor signaling pathway.

### **Conclusions:**

VEGF and squalamine synergism appears to be a phenomenon *in vitro* and its *in vivo* synergism is more difficult to demonstrate due to severe toxicity of delivery of VEGF to tumor tissues in tumor-bearing animals. The concept to enhance tumor and endothelial cell death using angiogenic modifiers however received support



by the application of squalamine immediately after castration. Based on immunohistochemical data, it appears that tumor cells overexpress VEGF receptor, flt-1 and specific integrin isotype, such as  $\alpha 6\beta 4$ , are responders. This part of the work is currently pursued in Dr. Mitch Sokoloff's lab with addition of radiation and squalamine as a new combination. This work will be further explored and will be the subject of a future human clinical trial.

#### References:

None

#### Appendices:

1. Figures 1-4.
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Figure 1

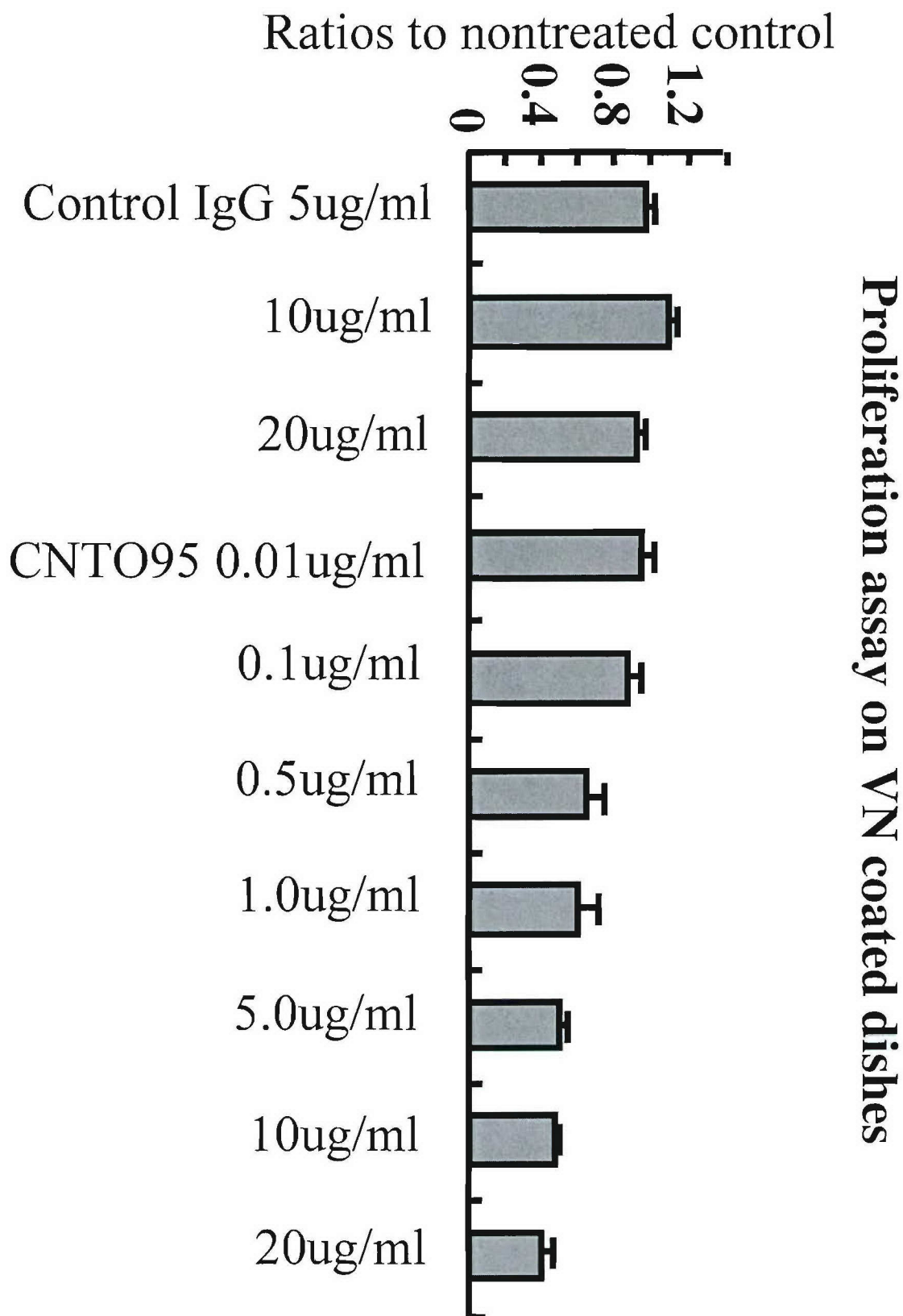




Figure 2

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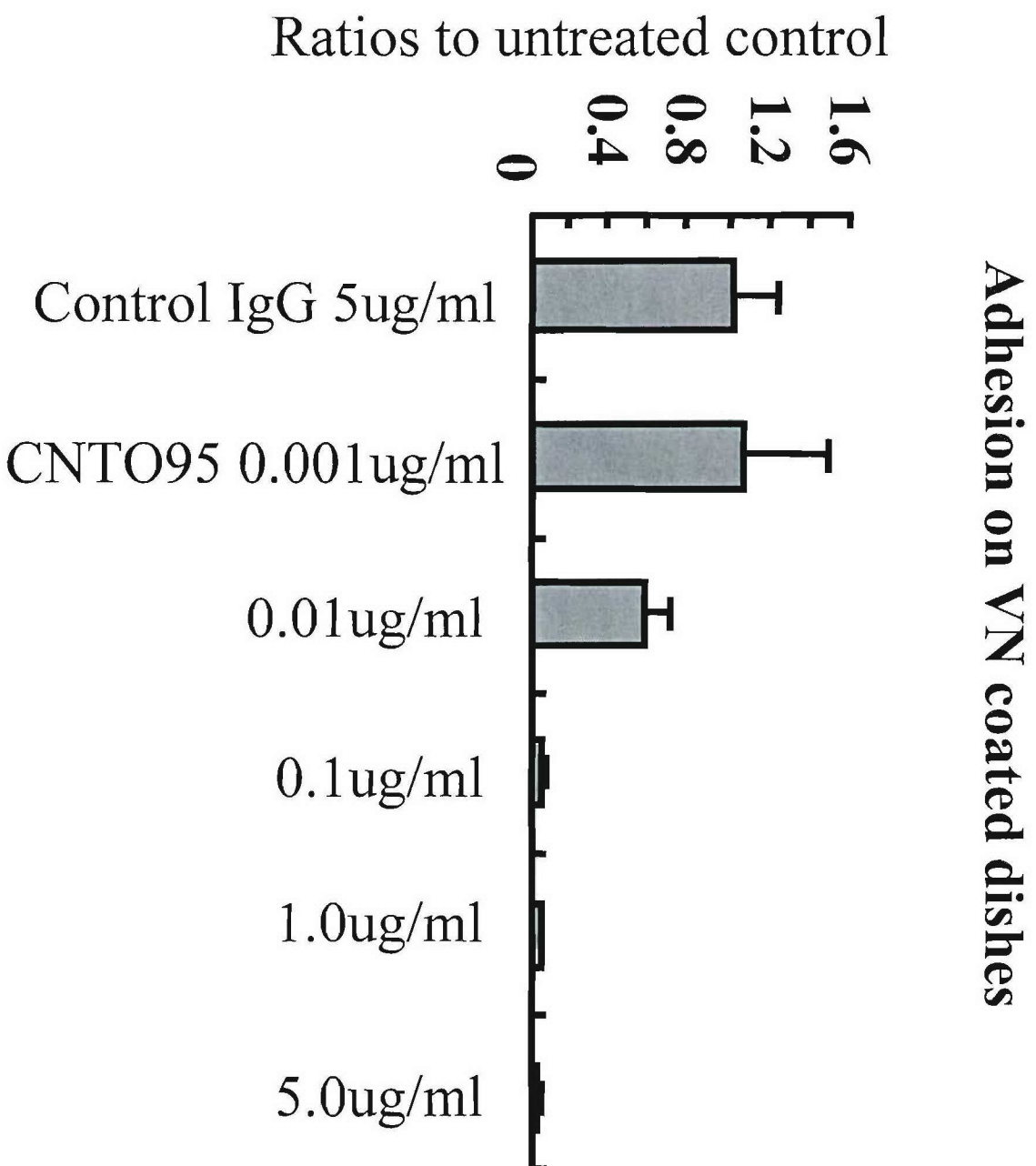


Figure 3

# Migration on VN coated dishes

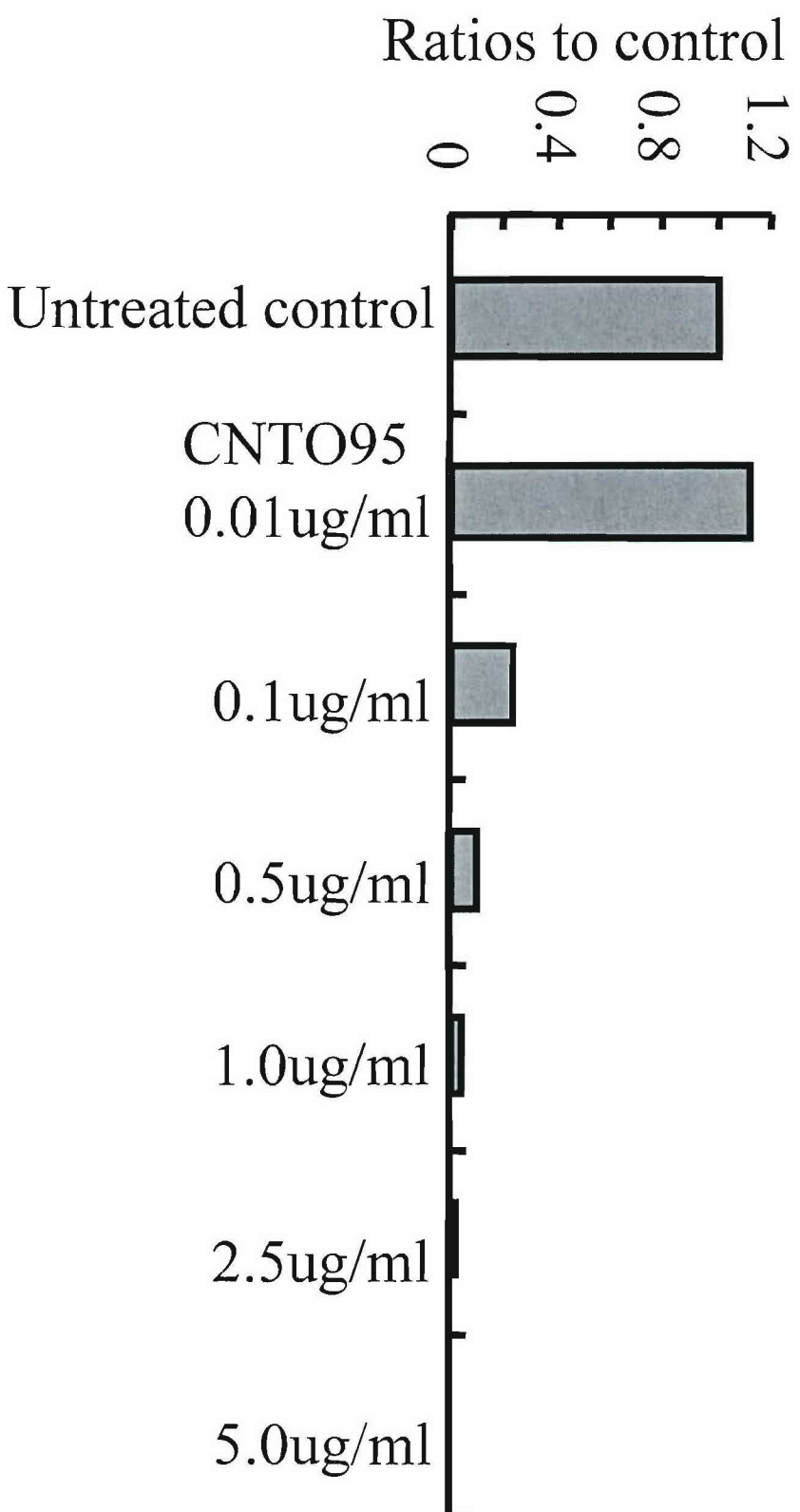
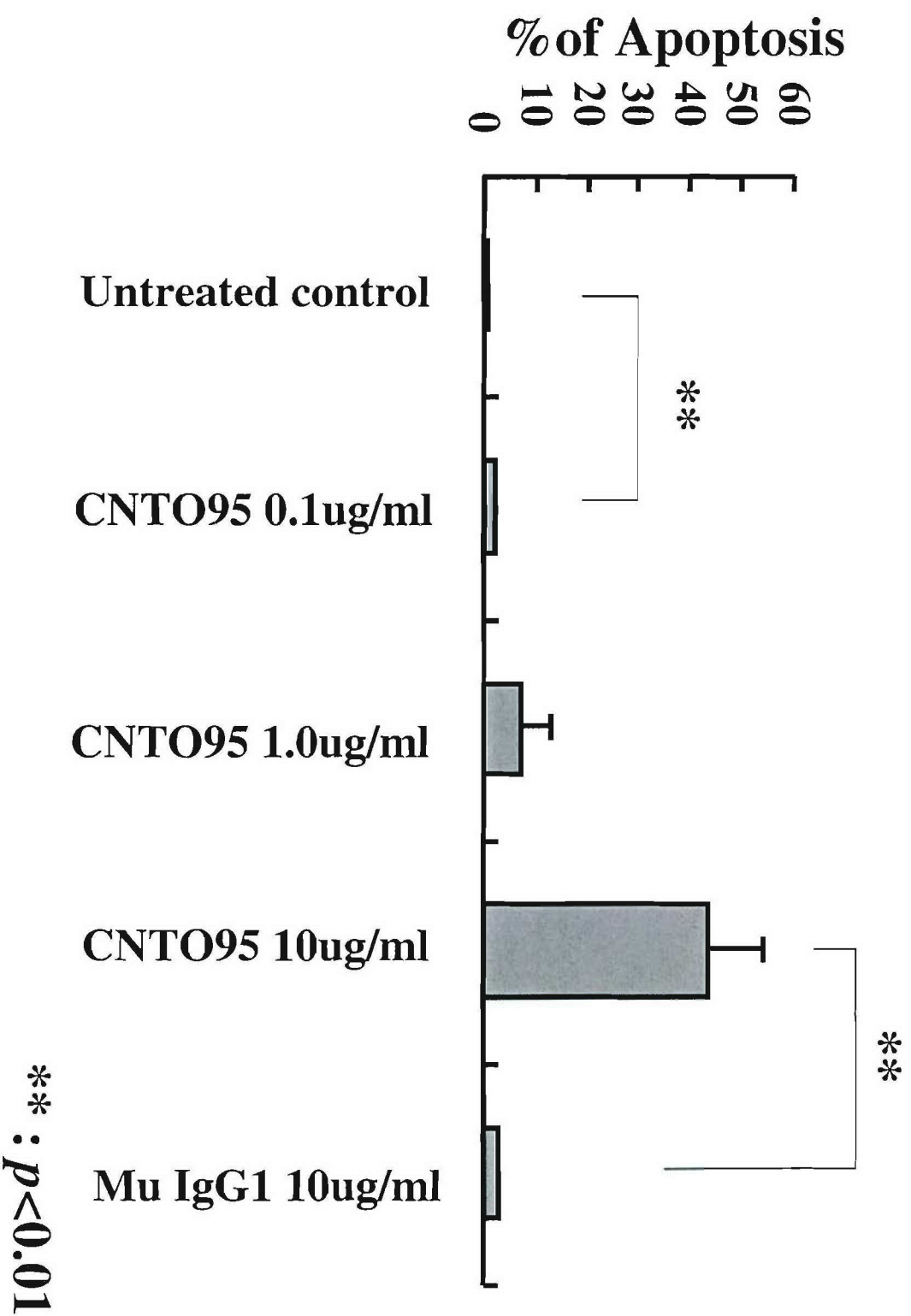


Figure 4

TOHRU MIYAGI et al.

# CNTO95 induce apoptosis





## Targeting angiogenic pathways involving tumor–stromal interaction to treat advanced human prostate cancer

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**Key words:** prostate cancer, stromal–epithelial interactions, anti-angiogenesis, intermittent androgen suppression

### Abstract

Interfering with and preventing tumor angiogenesis is an attractive therapeutic approach for treating cancer metastases. This commentary presents treatment strategies that may enhance the effectiveness of anti-angiogenic therapy by selectively targeting newly sprouting and immature vessels, inhibiting the production of angiogenic factors, and disrupting extracellular matrices. We propose several clinical paradigms, including hormonal ablation, intermittent androgen suppression, chemotherapy, and radiation therapy, that ‘injure’ nascent vasculature and interrupt the cancer cell–stromal relationship, thereby potentiating the efficacy of experimental anti-angiogenic agents. These stromal–epithelial interactions play an important role in the development, proliferation and dissemination of prostate cancer, as well as guiding the processes of tumor neovascularization. Successful utilization and targeting of tumor angiogenesis requires an increased understanding of tumor cell–stromal cell–endothelial cell relationships, most notably the intricate intracellular signalling cascades mediated by growth factors and the extracellular matrix.

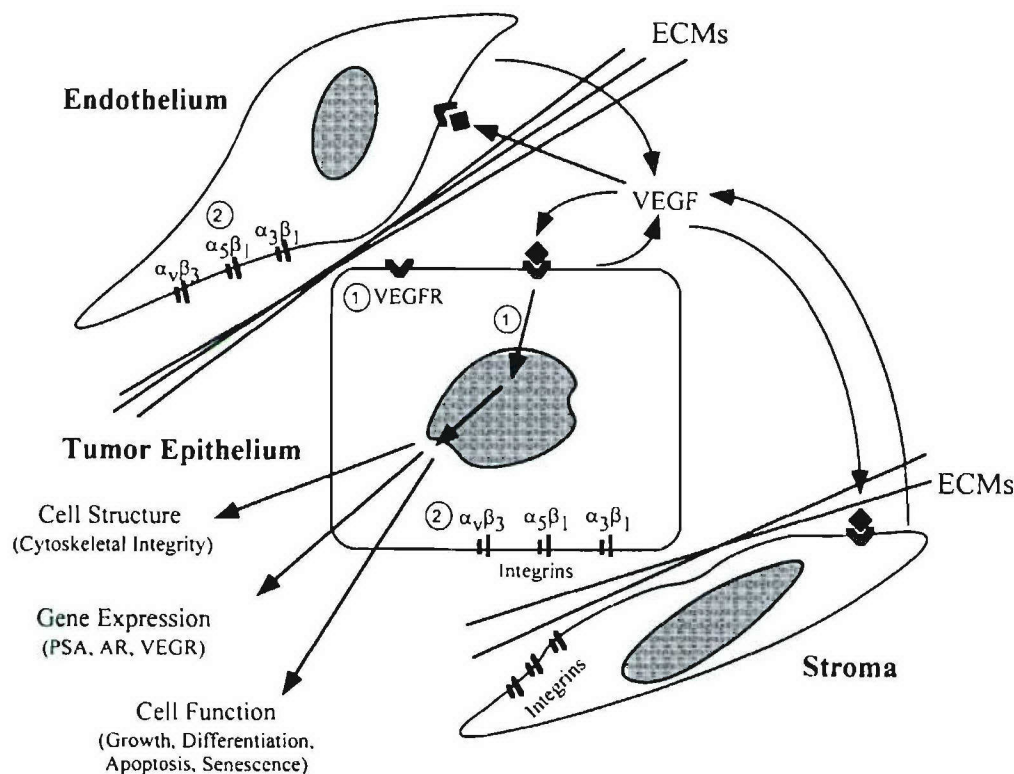
### Introduction

Despite advances in prevention and early detection, refinements in surgical technique, and improvements in adjuvant radio- and chemotherapy, the ability to cure many men with prostate cancer remains elusive. This is especially apropos to the successful management of metastatic and recurrent hormone-refractory disease. Clinical protocols using either androgen deprivation therapy or chemotherapeutic agents have shown some promise in treating advanced prostate cancer [1,2]. Unfortunately, the proportion and durability of complete remissions have been limited and new therapeutic approaches are desperately needed.

Stromal–epithelial interactions are paramount to the development, proliferation, and spread of prostate cancer. Studies in our laboratory have established that a bi-directional relationship between tumor cells and their surrounding stroma contributes to the growth and dissemination of prostate cancer [3–5]. These mesenchymal–epithelial interactions are responsible for maintaining the functional integrity of the

normal adult prostate gland. Irregularities in the constituents of the stromal–epithelial milieu or aberrations in their interactions can induce genomic instability, enhance tumor cell proliferation, and drive both metastatic spread and progression to a hormone-refractory state. Consequently, novel therapeutic protocols are being developed that target not only prostate tumor epithelial components, but surrounding stromal and extracellular matrix (ECM) elements as well.

For a prostate cancer to grow and metastasize, endothelial cells from this surrounding stroma must be recruited to form an endogenous microcirculation to support the developing neoplastic mass [6,7]. Similar angiogenic processes are necessary at sites of metastasis if disseminated tumor cells are to become securely entrenched and, subsequently, propagate. Although prostate cancer cells produce inherent pro-angiogenic signals, integration of downstream signaling involving soluble factors and stromal and ECM components are critical to promoting and maintaining neovascularization [8].



**Figure 1.** Prostate tumor cells express both vascular endothelial growth factor (VEGF) and its receptors (simplified here as a single entity, VEGFR). Intracellular communication between tumor epithelium, endothelium, and stroma is known to occur with signals mediated by such cell surface receptors as the integrins (e.g.:  $\alpha_v\beta_3$ ), which confer cell–matrix interactions and the VEGF receptors (e.g.: Kdr/flk-1 and flt-1), which confer VEGF-induced intracellular signal activation. Resultant VEGF-VEGFR signaling is growth stimulatory for endothelial cells. In studies in our laboratory [11], we have demonstrated that VEGF has either no effect on or, surprisingly, can inhibit the growth of prostate tumor epithelial cells. Depicted in this illustration are two potential sites of interaction for an anti-prostate cancer anti-angiogenic agent: (1) VEGF – VEGFR interaction; and (2) extracellular matrix (ECM) – integrin  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  interaction. Interruption of these cell-signaling pathways could be potentiated in the absence of the host androgen milieu or subsequent to any other therapy that injures the stromal–epithelial milieu and, hence, will adversely affect tumor cell survival in the stromal microenvironment. (Illustration courtesy of Song-Chu Ko, M.D., Ph.D.) [PSA, prostate-specific antigen; AR, androgen receptor].

Anti-angiogenic therapy has been demonstrated to be effective in several preclinical solid tumor animal models [9,10]. Moreover, since stromal factors can affect the multistep processes of both carcinogenesis and neovascularization, many of the factors involved in tumor angiogenesis are also associated with prostate cancer proliferation, progression, and dissemination. Hence, these stromal factors and interactions present an attractive therapeutic target for the treatment of hormone-refractory prostate cancer (see Figure 1). This commentary will review

the importance of prostate tumor cell–stromal cell interactions in tumor angiogenesis. In addition, we will conjecture how conventional therapies directed at interfering with the prostate tumor–stromal cell relationship may influence the extracellular milieu in such a manner that the anti-neoplastic effects of putative anti-angiogenic agents are enhanced. Finally, we will postulate how combining these two therapeutic approaches may improve our capacity to successfully manage men with advanced and metastatic prostate cancer.



### Stromal-epithelial cell injury may potentiate anti-angiogenic therapy

We have recently demonstrated that *squalamine*, an anti-angiogenic sterol isolated from shark liver, has potent anti-prostate cancer activity [11]. When squalamine was applied concomitantly with androgen withdrawal in a human prostate cancer xenograft model, an absolute and lasting eradication of both PSA and subcutaneous tumors was achieved. No such result was observed in intact tumor-bearing animals or in tumor-bearing animals treated with squalamine post-castration, yet subsequent to the appearance of androgen independent lesions. Immunohistochemical staining of responding and non-responding tumors indicated that combined squalamine and castration substantially diminished integrin  $\alpha_v\beta_3$  expression. Additional histologic data established that squalamine's actions were most potent in preventing proliferation of the freshly sprouting, phenotypically immature blood vessels that, we believe, emerge in the tumor tissue as it acquires hormone independence. This finding corroborated prior studies which have determined that squalamine's efficacy is significantly enhanced when tumor vasculature and cancer cells are 'injured' immediately antecedent to its application [12,13].

Accordingly, we have developed two theories to explain the powerful anti-angiogenic as well as anti-prostate cancer effects of coincident androgen ablation and squalamine administration. First, androgen deprivation, may so effectively 'stun' the stromal-epithelial environment that normally prevalent and active pro-angiogenic factors are diminished, inactivated, or eradicated. At this juncture, the diminution of angiogenic forces may be sufficient enough that the otherwise ineffectual squalamine (if used in the absence of hormone-ablation) is consequently rendered potent. Alternatively, during the immediate post-orchietomy rejuvenated proliferative phase (when stromal-mediated and autonomous epithelial tumor cell growth is actively acquiring hormone independence) the cellular and vascular architecture may become somewhat pliable and plastic, predisposing these precarious cells to either anti-angiogenic or cytotoxic effects of squalamine.

These interesting observations and speculations have led us to develop the following hypothesis: that the effectiveness of anti-angiogenic agents can be increased when applied coincident with other

therapeutic interventions that injure components of the stromal-epithelial milieu. This premise is particularly plausible considering that most pro-angiogenic factors are located within the stromal milieu, either as soluble or ECM-bound factors, which affects survival of tumor epithelium.

### Tumor-stromal interactions and angiogenesis

The outcome of a patient with prostate cancer ultimately depends upon the tumor's capacity for unhindered growth, local invasion, and the establishment of distant metastasis. Local factors, produced by mesenchyme, epithelial cells, or as a consequence of bi-directional mesenchymal-epithelial interactions between prostate tumor and stromal cells, are necessary for such proliferative, invasive, and migratory events [14].

Numerous cytokines and growth factors have been implicated in either enhancing or impairing a given prostate tumor's inherent tumorigenic and metastatic phenotype [15]. While some act directly upon the tumor cells, others influence prostate tumor cell proliferation by modulating their interactions with the extracellular matrix interactions through either soluble or matrix-associated signaling. This can significantly alter tumor cell heterogeneity with the propensity of selecting androgen-independent and metastatic variants.

### Angiogenesis

Angiogenesis refers to the formation of new blood vessels from pre-existing, nascent vasculature. It is a multistep sequential process involving the recruitment and proliferation of endothelial cells, their subsequent migration to the tumor mass, morphogenesis into a tubular structure, and maturation into a stable structure [16,17]. It is important to note that the structure of tumor vessels differ from those of normal tissues, especially with regard to cellular composition, tissue integrity, vascular permeability, and regulation of cell proliferation and apoptosis [18]. It is presumed that these many differences may impart selective susceptibility of tumor vessels to the effects of anti-angiogenic agents.

The establishment and maintenance of such a vascular supply is imperative to prostate carcinogenesis and involves the cooperation of a variety of molecules either constituting or inhabiting the ECM. A variety of



growth and survival factors present within the extracellular matrix have been associated with angiogenesis [19–21]. These include, but are not limited to, TGF- $\alpha$  and  $\beta$ , b-FGF, IGF-1, EGF, HGF/SF, PDGF, TNF- $\alpha$ , VEGF, and IL-6 and 8. As it is beyond the scope of this commentary to discuss all of these factors in much detail, we will briefly mention the best characterized and most studied soluble pro-angiogenic factors of the prostate cancer cell-ECM milieu: *vascular endothelial growth factor* (VEGF) [22–24].

VEGF is a potent stimulatory factor for angiogenesis and is a highly specific mitogen for vascular endothelial cells. It has been implicated in promoting prostate carcinogenesis and metastasis, as well as angiogenesis. It is commonly accepted that VEGF influences tumor growth indirectly, through angiogenic activity, although a direct effect has never been excluded and more recent data suggests a non-angiogenic mechanism (see below). VEGF has also been shown to stimulate cell migration, implicating it in tumor metastasis as well as angiogenesis. VEGF is expressed by prostatic cancer epithelium, and expression positively correlates with increasing grade and tumorigenicity [25–28]. In the LNCaP cell line, VEGF appears to be androgen regulated, suggesting that hormone ablation therapy may act, in part, through the inhibition of VEGF production [29,30]. These results, however, have been contradicted in other studies using different prostate cancer cell models (see below).

#### *Integrin signaling*

Although the ECM is an important determinant in tumor cell growth, survival, and dissemination, its potency depends upon successful tumor cell-ECM signaling. Cell-ECM interaction is signaled primarily through integrins, which are comprised of over twenty combinations of  $\alpha$  and  $\beta$  heterodimers [14,31]. ECM-integrin signaling has been broadly implicated in regulating angiogenesis, as well as tumor cell motility, migration, and metastasis [14,20]. In the LNCaP/C4-2 progression model of human prostate cancer metastasis [32], we have documented a functional integrin switch from  $\alpha_6\beta_4$  to  $\alpha_3\beta_1$  and  $\alpha_v\beta_3$  during prostate cancer progression. This switch appears early in the progression to androgen independence and has been confirmed biochemically by immunoprecipitation of biotinylated prostate tumor cells followed by subsequent western blotting of these products using avidin-conjugated peroxidase antibody. We have further demonstrated

that integrin  $\alpha_v\beta_3$  preferentially attaches to vitronectin and osteopontin, key components of bone matrix, and that osteopontin is overexpressed in pathologic specimens from men with hormone-refractory prostate disease. This is likewise associated with a coexistent overexpression of integrin  $\alpha_v\beta_3$ . Osteopontin, a potent autocrine and paracrine growth factor, is secreted by both prostate cancer epithelial and bone stromal cells, exerting a direct stimulatory effect on prostate epithelial cell proliferation *in vitro* [33].

Integrin  $\alpha_v\beta_3$  has a prominent function in tumor angiogenesis [31,34]. Furthermore, integrin  $\alpha_v\beta_3$  activity is modulated by several growth factors that reside in the ECM, such as VEGF. In chick chorioallantoic membrane (CAM) assays, angiogenesis can be disrupted by treatment with either a cyclic peptide or monoclonal antibody antagonist to  $\alpha_v\beta_3$  [35]. *In vivo* studies have demonstrated that tumor growth can be inhibited when integrin  $\alpha_v\beta_3$  expression is likewise constrained [36]. Since integrin  $\alpha_v\beta_3$  influences angiogenesis, reorganization of cytoskeletal structures, basement membrane attachment, and cellular proliferation, a switch to integrin  $\alpha_v\beta_3$  in prostate cancer may provide advantages to growth of androgen independent tumor cells at both primary and distant sites.

Clinically, the relationship between prostate cancer and angiogenic factors remains controversial [28,37–40]. In most studies, increases in tumor angiogenesis correlate with Gleason grade and with progression after prostatectomy. Angiogenic factors are also associated with a heightened metastatic phenotype and with poor prognosis. Furthermore, quantitation of tumor angiogenesis, based on microvessel density, appears to be a promising technique for estimating the extensiveness and aggressiveness of a given prostate tumor as well as predicting its response to various forms of treatment.

#### **Combining conventional and anti-angiogenic approaches to treat men with advanced prostate cancer**

Radical prostatectomy can cure patients with localized prostate cancer and its use in treating such tumors in younger and healthy patients is generally undisputed [41]. Nonetheless, almost 30 percent of patients with pathologically organ-confined cancer will experience an early relapse with recurrent disease despite successful treatment of the primary lesion [42]. Furthermore, current screening modalities fail to identify a significant subset of patients with locally-invasive tumors.

Recent studies report that up to 50 percent of patients who were thought to have organ-confined lesions were discovered to be understaged subsequent to surgery [43–45]. As a result, the majority of men with prostate cancer will eventually develop disseminated disease [46]. In addition to causing severe pain and morbidity, such metastatic disease is the primary cause of death in men with prostate cancer [47]. Androgen ablation therapy is the most widely accepted therapy for men with metastatic cancer. Because of its limited duration, however, certain chemotherapeutic agents have been incorporated in the treatment of advanced, hormone-refractory disease. Furthermore, radiation therapy (with or without hormonal therapy) is commonly used to treat locally-invasive lesions that are felt to be incurable by surgical means.

With regard to prostate cancer, androgen ablation therapy, chemotherapy, and radiation therapy share two common traits. First, by themselves, they customarily behave as temporizing agents resulting in disease remission, but are generally ineffective in curing advanced disease. Second, in addition to directly damaging prostate cancer cells, each of these treatment modalities induces injury to the surrounding stroma and extracellular matrix. In fact, studies demonstrating improved outcomes in men with prostate cancer after treatment with combined radiation and androgen ablation therapy attribute these findings, in part, to the resultant interference with the stromal–epithelial relationship [48]. Furthermore, p53, which can act as a radiosensitizer, may enhance the efficacy of radiation therapy via its anti-angiogenic properties, such as inducing expression of the anti-angiogenic ECM component, thrombospondin-1 [49,50]. Because of their individual and independent abilities to injure components of the extracellular milieu, androgen ablation therapy, chemotherapy, and radiation therapy are ideal therapeutic approaches to investigate for use in concert with anti-angiogenic agents to treat men with, or at risk for developing, advanced prostate cancer.

Taking all three of these treatment modalities into consideration, we can now reiterate our hypotheses as to how androgen ablation therapy, chemotherapy, and radiation therapy may induce sufficient injury to the stromal–epithelial environment that the effects of the subsequent utilization of anti-angiogenic agents would be potentiated. First, damage to the homeostatic cellular components of the stromal–epithelial milieu might decrease, or even completely suppress, the secretion of soluble pro-angiogenic factors and intracellular

signaling of pro-angiogenic components, inhibiting many of the steps required for neovascularization. Such alterations in this constituency could greatly increase the sensitivity of the tumor to anti-angiogenic therapy. Second, injury to the vascular endothelial cells within the stroma could result in the immediate destruction of the tumor vasculature (resulting in the cessation of blood flow to the cancer cells) or weaken the vessels sufficiently that their susceptibility to attack by a second (anti-angiogenic) agent is increased. Furthermore, damage to established vessels might induce the formation of new vasculature to nourish and sustain both the tumor mass and the surrounding tissues, or alter the phenotype incipient vasculature so that it acquires the more immature characteristics of newer vessels. It is generally accepted that these younger, more immature vessels are most-susceptible to anti-angiogenic insult [51]. Third, immediately following intervention, some stromal and epithelial tumor cells are likely to overcome their injuries and begin to initiate repair pathways, in which they recapitulate a phenotypically younger and more unstable configuration. As the cellular architecture becomes increasingly precarious, these cells are more apt to be affected by the anti-angiogenic agents.

#### *Androgen ablation therapy*

Our data from the LNCaP-castrate xenograft model confirmed that squalamine's actions were most potent on the freshly sprouting, immature blood vessels that, we believe, developed during a rejuvenated proliferative phase of those prostate tumor cells acquiring hormone independence. The effect was independent of serum VEGF levels. There is ample evidence that androgen application can stimulate vasculogenesis whereas androgen deprivation can inhibit neovascularization, allegedly the result of increased or decreased VEGF production (respectively). Folkman has demonstrated that VEGF production by LNCaP cells is under tight regulation by androgen and that androgen withdrawal inhibited hypoxic induction of VEGF [52]. Isaacs and associates have demonstrated that the activity of Linomide, an oral anti-angiogenic agent which has demonstrated effectiveness in suppressing human prostate cancer in preclinical animal studies, was potentiated by concurrent androgen ablation, presumably due to down-regulation of VEGF [30].

It was recently demonstrated that prostate gland growth in a rat model was regulated by the vascular



endothelium, which, accordingly, was itself controlled by testosterone stimulation [53]. In this study, testosterone stimulation in castrated animals caused an escalation of endothelial proliferation and vessel development, antecedent of glandular tissue regrowth, allegedly the result of increased VEGF production. In the Dunning prostate adenocarcinoma model, however, castration had little effect on VEGF production, despite enhancement of VEGF flk-1 receptor expression and involution of tumor vasculature [54]. This critical observation suggests that castration can affect angiogenesis independently of VEGF production and receptor status and allows for the identification of multiple pathways of anti-tumor anti-angiogenic activity. The presence of many such pathways implies that in prostate cancer, a synergistic therapeutic approach could be developed by using androgen deprivation therapy along with other anti-angiogenic agents, thereby enhancing the effectiveness of each individual treatment modality [30].

Additional evidence to support using such a combined approach to managing human prostate cancer can be found in a recent article by Jain et al. [55]. Using the Shionogi tumor model, androgen ablation initially resulted in tumor involution, in which endothelial cells underwent apoptosis before neoplastic cells. Soon after castration, however, the regressing vessels began to exhibit changes in phenotype. Subsequently, they began to produce large quantities of VEGF and both neovascularization and tumor regrowth resulted. We propose that by working through one of the several putative anti-tumor/anti-angiogenic pathways postulated above, the application of an anti-angiogenic agent coincident with castration could inhibit this neovascularization and tumor regrowth, hence prolonging cancer remission.

#### *Intermittent androgen suppression*

The above data suggest that by injuring the prostate tumor cell–stromal cell–ECM environment, castration induces degeneration of vascular structures. This appears to precede the apoptotic effects seen in the tumor cells themselves and can be independent of VEGF production. As the effects of castration wane, there is a resurgence of pro-angiogenic influences with resulting vasculogenesis. These vessels are young, immature, pliable, and most prone to being affected by the administration of anti-angiogenic agents. In this commentary, we have proposed using orchiectomy

in concert with anti-angiogenic therapy to maximize the potential of each to repress or, hopefully, eradicate prostate cancer. If castration by itself appears so promising as a neoadjuvant to anti-angiogenic therapy, intermittent androgen suppression (IAS) should, theoretically, have a more pronounced effect.

IAS has been applied in both pre-clinical models and in men with advanced prostate cancer in an attempt to prolong the anti-neoplastic effects of hormone ablation therapy [56–59]. The underlying biologic principle of IAS is straightforward. Androgen ablation inhibits proliferation of androgen-sensitive prostate tumor cells as well as inducing apoptosis. In the normal prostate, androgen-induced growth and castration-induced regression can be repeatedly cycled using androgen replacement and withdrawal, respectively, since normal prostatic epithelial cells do not acquire the ability to grow in an androgen-deficient environment. In prostate cancer, however, cells that escape the cytotoxic effects of hormone ablation emerge as a population of androgen resistant clones. This ultimately results in tumor growth and metastasis, with eventual death. Hormone-refractory tumor cells may result from the selection of pre-existing androgen independent cells resistant to apoptosis, or from upregulation of adaptive mechanisms to the androgen independent state. IAS is founded on the premise that with intermittent androgen exposure, tumor cells may delay their progression to an androgen-independent state. Conceivably, however, intermittent androgen exposure can also inhibit tumor cell growth through an androgen-repressed (or hypersensitivity to androgen) status [60]. Overall, IAS may restore apoptotic potential and delay the progression of prostate cancer cells to androgen-independence.

Few clinical trials with IAS have been completed to date. Some suggest slightly-improved survival rates, with substantial improvement in quality in life [57]. For our purposes, however, IAS offers an attractive opportunity to test our hypothesis that applying anti-angiogenic agents coincident with stromal injury and repair will enhance tumor kill. IAS involves cyclical androgen-induced growth and castration-induced regression. With each course, there is the same initial injury to the stroma and ECM seen with castration, however under the influence of hormone repletion, there is a resurgence of pro-angiogenic influences with resulting vasculogenesis. These are the young, immature, and pliable vessels most apt to being affected by the administration

of anti-angiogenic agents. By cyclically applying and withdrawing androgen, intermittent androgen blockade systematically injures tumor and vascular cells over a prolonged period of time. This repeated stress should prime them for the actions of an anti-angiogenic agent, resulting in the destruction of both tumor cells and the surrounding stroma.

#### *Chemotherapy and radiation therapy*

External beam radiotherapy, as well as brachytherapy, is frequently applied to the treatment of local and locally-advanced prostate cancer. Furthermore, radiation therapy is commonly used to treat symptomatic metastases. Radiation induces significant injury to both tumor and stromal cells [61,62]. There is often scarring and destruction of nascent vasculature, as well as damage to the surrounding stromal and ECM components. In both situations, much akin to androgen ablation therapy, the stromal-epithelial milieu initially experiences significant injury with damage to stromal cells as well as pro-angiogenic signals. With time, however, these cells attempt to repair the radiation-induced damage, and the stromal-epithelial compartments undergo neovascularization with the potential to support a tumor recurrence. Similarly, applying taxol and estramustine, two currently used agents in prostate disease and as a paradigm for other chemotherapeutic regimens, will similarly induce injury of both endothelial and epithelial tumor cells [63,64]. We propose that the application of an anti-angiogenic agent coincident with radio- or chemotherapy induced injury could inhibit stromal neovascularization and prevent tumor recurrence.

#### **Concluding remarks**

Anti-angiogenic agents have shown promise in several preclinical studies of prostate cancer. In our own experience squalamine, an aminosterol with anti-angiogenic properties, has demonstrated effectiveness when applied concomitant with castration. As many of the pro-angiogenic influences present in prostate carcinogenesis reside with the stromal-tumor cell-ECM environment, it is not surprising that castration, which induces widespread damage within the stroma (in addition to having direct cytotoxic effects), would potentiate the activity of an anti-angiogenic agent. In this commentary, we speculate that the potency of

anti-angiogenic agents will be most pronounced when applied in conjunction with other therapeutic modalities that maximally injure the stromal and ECM. For now, this includes intermittent androgen suppression, radiation therapy, and chemotherapy, but may, with time, be applicable to gene therapy and novel molecular approaches being developed for the treatment of both localized and advanced human prostate cancer.

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## REVIEW

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**Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting**

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**Abstract** Maintenance of cell and tissue homeostasis is dependent upon the dynamic balance of cell proliferation, differentiation, and apoptosis through interactions between cells and their microenvironment. The unique prostatic cellular phenotypes are induced and maintained by interaction between epithelium and adjacent stroma through intimate intercellular signaling pathways. In this article, we summarize current advances in the tumor-stroma interaction and its biologic and therapeutic implications. We specifically emphasize current studies of the possible factors driving the “vicious cycle” between stroma and emerging prostate tumor epithelial cells that may be responsible for carcinogenesis and metastasis to bone. Stroma responds both genotypically and phenotypically to tumor epithelium upon co-culture under 3-D conditions. Likewise, the emerging carcinoma responds to stromal signals that drive progression to malignancy. A vicious cycle mediated by soluble and insoluble molecules secreted by tumor cells and stroma appear be the critical factors supporting and sustaining tumor colonization in bone. Co-targeting tumor and stroma with therapeutic agents has yielded promising results both in pre-clinical models of prostate cancer and bony metastasis and in clinical trials of patients treated with a dual tumor and stroma targeting strategies. In conclusion, understanding and targeting the interaction of the tumor and its stromal microenvironment may improve the prognosis, reduce the suffering and increase the survival of patients with advanced cancer metastasis.

**Key words** prostate cancer · bone metastasis · stromal-epithelial interaction · molecular targeting · vicious cycle · cytokine · extracellular matrix (ECM)

**Introduction**

This review will first elucidate the molecular mechanisms underlying prostate tumor-stroma interaction, involving prostatic stromal cells at the primary site and osteoblasts and osteoclasts at bone metastasis sites, and then discuss new opportunities for therapeutic targeting of localized and disseminated human prostate cancer.

Cell and tissue homeostasis reflects a dynamic balance of cell proliferation, differentiation and apoptosis (Frisch and Screaton, 2001). Consistent with this concept, primary and immortalized non-transformed human prostate epithelial cells require adhesion to an extracellular matrix (ECM) to maintain their polarity, growth, survival, and migratory characteristics and expression of tissue-specific proteins. These properties are unique organ-specific phenotypes conferred and maintained by interaction between epithelium and adjacent ECM secreted primarily by the stroma through intimate intercellular signaling pathways. Epithelial cells, the major target for adult cancer, exist in contiguous sheets composed of organized, polarized cells circumscribed by a basement membrane that separates the epithelium from the stroma. Numerous cell types are found in the epithelial and stromal compartments, including luminal, basal, and neuroendocrine cells in the epithelial tissue compartment and smooth muscle, fibroblast, endothelial, neuroendocrine, neural, and inflammatory cells in the stromal tissue compartment. Intercellular interaction between these cell types, mediated by soluble factors and insoluble ECM, will determine the growth and differentiation potentials of the entire organ.

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Homeostasis of normal organs such as prostate and breast is maintained through reciprocal interactions between epithelial cells and their surrounding stroma with minimal proliferation of either cell type. Disruption of the homeostatic interaction between epithelium and stroma could initiate and promote carcinogenesis. In these instances, carcinogenic insults may trigger additional genetic changes in the epithelial cell compartment over and beyond the inherited traits, through increased genomic instability and decreased DNA repair and apoptotic signaling. Altered epithelial cells may trigger stromal reactions that, in turn, confer reciprocal signal cascades in tumor epithelium to promote further carcinogenic processes. Ultimately, reciprocal tumor-stroma interaction culminates in the increased migratory, invasive, and metastatic behavior of cancer cells.

### Prostate carcinoma-stroma interaction

Prostate epithelium and stroma are sites for the development of benign and malignant diseases of the prostate. Recent evidence suggests that prostate epithelium and stroma interact in a highly organ-specific, androgen-dependent and temporally-related manner. We discuss below the role of prostate fibromuscular cells in prostate tumor growth and progression, the reciprocal stromal reactions to prostate tumor epithelium that create a "vicious cycle" between stroma and epithelium ultimately driving tumor epithelium to develop benign and malignant prostate diseases, the potential factors that may mediate these interactions, and the role of early prostate inflammatory atrophy in the development of benign prostatic hyperplasia and prostate cancer.

#### Role of prostate fibromuscular stromal cells in prostate tumor growth and progression

In 1970, Professor L. M. Franks described the requirement of prostate cancer fibromuscular stromal cells for the growth and survival of primary human prostate epithelial cells in culture (Franks et al., 1970). We tested the significance of this original *in vitro* observation in our laboratory by both *in vitro* cell co-culture and *in vivo* co-inoculation of tumor cells and stromal cells in immune-compromised mouse models for the growth of human and rat prostatic tumors as xenografts. A series of reports demonstrated that the growth of benign and cancerous prostate epithelial cells *in vivo* was enhanced markedly by the co-presence of organ-specific and/or cancer-associated stromal cells (Chung et al., 1989; Camps et al., 1990; Gleave et al., 1991; 1992). These studies were confirmed by other laboratories which showed that prostate tumor growth *in vivo* could be accelerated by cancer, but not by benign tissue-associated stromal fibroblasts (Olumi et al., 1998; 1999; Wong and Wang, 2000).

Further, the androgen-independent and metastatic progression of human prostate epithelial cells can be promoted by co-inoculating a marginally tumorigenic human prostate cell line, LNCaP, with a human bone stromal cell line derived from an osteosarcoma *in vivo* (Thalmann et al., 1994; 2000; Wu et al., 1994; 1998). By a series of manipulations of chimeric LNCaP tumor growth *in vivo* under the influence of bone stromal cells, either in the presence or absence of androgen, the derivative LNCaP sublines C4-2 and C4-2B acquired the ability to become androgen-independent and metastatic, as exhibited by their behaviors in immune compromised mice. To ascertain that cell-cell contact rather than unknown factors from the host were responsible for conferring tumorigenic and metastatic potential to the parental LNCaP cells, we co-cultured LNCaP cells with either prostate or bone stromal cells under 3-dimensional (3-D) conditions and observed similar permanent phenotypic, genotypic and behavioral changes of the parental LNCaP cells, as revealed by their ability to form tumors in castrated mice and acquired ability to metastasize to distant organs including bone (Ozen et al., 1997; Pathak et al., 1997; Rhee et al., 2001).

These results taken together suggest that tumor stroma can confer "inductive" or "adaptive" cues to the responding tumor epithelium and is directly responsible for the altered behavior of tumor epithelium. However, tumor-stroma interaction is reciprocal. Not only can stroma "induce" or "select" the phenotypic and genotypic changes in tumor epithelial cells, tumor epithelium can also induce genetic and phenotypic changes in stroma after tight association *in vivo*. It appears that **both** tumor and stroma are involved in controlling tumor growth or "take" by the host and the subsequent progression of tumor epithelium to androgen-independence and acquisition of local invasive and distant metastatic potential in experimental models of human prostate cancer. Figure 1 depicts a model of the multi-step nature of this interaction. Genetically and phenotypically altered epithelial cells induce a stromal reaction that, in turn, induces a reciprocal epithelial reaction. The serial interactions form a "vicious cycle" that drives epithelial cancer progressive to androgen-independent local invasion and distant metastasis.

Through growth factor activation, changes in ECM can be elicited, which can cause epithelial cells to lose their apical-basal polarity and thus assume a less well differentiated state (Bissell and Radisky, 2001). This dramatic alteration of epithelial cell phenotype can lead to increased cell proliferation and tumorigenesis (Reichmann, 1994; Naishiro et al., 2001).

However, the specific molecules responsible for tumor-induced changes in the microenvironment and the reciprocal modifications of the tumor by its microenvironment are largely unknown, as are the inter- and intra-cellular pathways that result from these influences. Dissecting the components of the stroma requires model



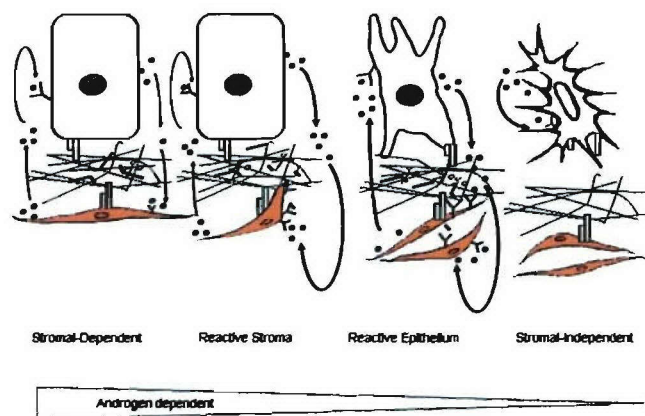


Fig. 1 "Vicious cycle" of prostate cancer bone metastasis. This figure describes the growth factor and extracellular matrix-mediated activation of transcription factors, which control the matrix proteins, and MMPs activation. The vicious cycle may be initiated first by the presence of growth factor/ECM milieu in the prostate cancer which up-regulates key transcription factors that modulate matrix and metalloproteinase expression in stromal cells. Increased expression of chemokines, cytokines, and transcription factors can activate additional growth factors and ECM pathways, which drive prostate tumor cells to a more invasive and malignant state.

systems in which a single variable can be manipulated and assessed. In contrast to tumor-associated stroma, normal stromal cells have a low proliferative index, probably secrete only the factors necessary to maintain normal tissue function (Manabe and Owens, 2001), and appear to be less responsive to inductive cues elaborated from normal epithelium.

### Stromal reaction to tumor epithelium

Since stromal cells from normal adult tissues are less inductive, or often non-inductive, the experimental data imply that stromal cells exposed to tumor epithelium could be "activated" and acquire an inductive potential to drive the subsequent neoplastic processes. This suggestion is supported by several lines of evidence.

First, morphologic "desmoplastic" stromal response to tumor epithelium often occurs around either primary or metastatic tumor epithelium (Thompson et al., 1993; Nemeth et al., 1999; Tuxhorn et al., 2001; 2002). A desmoplastic stromal response is characterized by increased proliferation of fibromuscular stromal cells and enhanced deposition of extracellular matrices (ECMs) surrounding tumor epithelium. This active process could be viewed as a part of the host-defense mechanism to curtail or restrict tumor expansion. Conversely, this reaction and accompanying increased stromal cell number could provide a "fertile soil" supporting the growth and invasion of tumor epithelium through the increased production by stromal cells of growth factors and stroma-associated ECMs. In addition, stromal cells are the

major production sites of metalloproteinases, which increase ECM turnover and are thought to be critical to the invasive property of tumor epithelium. Thus, global changes in the tumor microenvironment could provide selective growth and survival advantages for certain tumor cell clones, particularly in androgen-deprived conditions.

Second, stromal reaction to tumor epithelium may be irreversible, if the reacting stromal cells receive an "inductive cue" from tumor epithelium to undergo trans-differentiation, whereby stromal fibroblasts adjacent to tumor epithelium convert both morphologically and phenotypically to myofibroblasts (Ronnov-Jessen et al., 1995; Elenbaas and Weinberg, 2001; Tuxhorn et al., 2001, 2002).

Transition of stromal fibroblasts to myofibroblasts with increased expression of vimentin pro-collagen type-I and tenascin has been observed (Tuxhorn et al., 2001). Using the laser capture microdissection (LCM) technique, genetic aberrations were detected in the fibromuscular stromal compartment surrounding tumor epithelium, further supporting the reciprocal nature of the tumor-stroma interaction (Moinfar et al., 2000). Although the mechanisms of the genetic and phenotypic response of stroma to adjacent tumor epithelium are currently unclear, possible mechanisms include: a) Transition or inter-conversion of epithelium to stroma (Wernert et al., 2001); b) Irreversible induction of stromal changes, both at the morphologic and the biochemical levels, by soluble and insoluble factors secreted by tumor epithelium; c) Selection of previous existing clones of stromal cell populations and preferential expansion of these clones based on their proliferative and survival advantages (Singer et al., 1995; Tso et al., 2000); d) The combination of b and c above; that is, after prolonged "adaptation" to a tumor-associated stromal microenvironment, permanent genetic changes may occur in the stromal cell population through a poorly understood "adaptive mutation" mechanism (Laval and Laval, 1984; Chung, 1995).

### A "vicious cycle" between prostate stroma and tumor may be responsible for carcinogenesis in primary prostate cancer

Studies using tissue and cell recombination models demonstrate that growth and differentiation of the prostate gland depends on reciprocal cellular interaction between prostate epithelium and its adjacent stroma (Chung et al., 1991; Cunha et al., 1996; Wong et al., 1998; Wong and Wang, 2000). Evidence also suggests that androgen receptor in the stroma rather than in the epithelium may be critical for conferring the growth and differentiation functions of the prostate gland (Cunha and Chung, 1981; Thompson and Chung, 1984). When normal prostate epithelium (Zhau et al., 1994; Chung, 1995) or urothelium (Zhau et al., 1994) was used in these studies, the



inductive fetal urogenital mesenchyme determined the ultimate size of the tissue-tissue recombinant. However, when prostate tumor tissues (Chung et al., 1984; Miller et al., 1985) or tumor cells derived from the prostate (Gleave et al., 1991; 1992) or urinary bladder (Zhau et al., 1994) were used in the experiments, the growth of the tissue-tissue or tissue-cell recombinants was uncontrolled and never reached a state of homeostasis.

One interpretation of these results is that signaling between tumor and stroma is aberrant and resembles a "vicious cycle" where a dysfunction of cytokine trafficking exists between tumor and host cells (Mundy et al., 2001; Tester et al., 2002). There are several possible mechanisms for the activation of a vicious cycle between tumor and stroma: 1) tumor cells secrete putative cytokines, growth factors and/or extracellular matrices that alter the morphology and gene expression of surrounding stroma such that the altered stroma becomes highly inductive and reciprocally induces the growth and gene expression of tumor epithelium, thus initiating the vicious cycle. Rowley and collaborators provided evidence that stromal fibroblasts surrounding tumor epithelium underwent trans-differentiation to become a morphologically and biochemically distinct population of myofibroblasts (Rowley, 1998; Tuxhorn et al., 2001; 2002). Interestingly, they have shown that this type of stromal response to tumor epithelium can predict PSA-free survival in patients with prostate cancer. 2) Tumor cells secrete soluble factors that act in an autocrine manner to promote the vicious cycle regardless of the surrounding stroma. Under certain stress and androgen conditions, increased growth factors, such as vascular endothelial growth factor (VEGF) production by tumor cells, have been observed (Wong et al., 1998; Jackson et al., 2002). Increased VEGF was shown to induce more oxygen stress and initiate the vicious cycle by promoting more VEGF production by tumor cells, eventually causing an accumulation of neovasculature surrounding the tumor epithelium (Ferrer et al., 1997; 1998; Burchardt et al., 2000; Arbiser et al., 2002; Colavitti et al., 2002). 3) The intrinsic genetic instability of tumor cells can be promoted by tumor-microenvironment interaction (Rhee et al., 2001; Tlsty, 2001). It is conceivable that increased cytogenetic changes, with loss or gain of growth control genes, could fuel additional genetic instability not only of tumor cells *per se* but also of their surrounding stroma (Moinfar et al., 2000).

### **Potential factors responsible for activating prostate carcinogenesis and driving the "vicious cycle" of prostate stroma and tumor**

#### **Integrins**

Integrins are important in prostate cancer progression and metastasis. The major role of integrins in cancer is

the "outside-in" pathway, in which integrin activation induces cancer cell migration and invasion. Integrins also cooperate with growth factors to promote cell proliferation. When adherent tissue cells are released from their surrounding extracellular matrix, they forfeit survival signals and undergo apoptosis (Porter and Hogg, 1998). In addition to interacting with stromal cells or ECM, integrins can also form cis associations with other receptors on the same cell, forming multi-receptor complexes. These complexes recruit signaling molecules to sites of cell-cell or cell-matrix adhesion, such as focal complexes and focal adhesions (Edlund et al., 2001). Integrins also play a crucial role in regulating the actin cytoskeleton at the site of contact with ECMs. Although the detailed pathway is still not very clear, data show that once integrins receive signals from ECMs they can turn on other genes such as  $\alpha$ -actin, talin, vinculin and vasodilator-stimulated phosphoprotein. Signaling through Rho family pathways could activate Cdc42, Rac and Rho genes which further turn on downstream signaling pathways such as the calpain and JNK pathways. Integrins also could regulate the activation of the focal adhesion kinase pathway, Src protein tyrosine kinase, and paxillin, which are important in the remodeling and turnover of adhesion complexes (Martin et al., 2002).

Modulation of integrin activation is closely linked to gene expression, cell cycle progression and cellular behaviors, such as cell motility, migration, and survival under various physiologic and pathologic conditions. The increased expression of  $\alpha 3$  and  $\beta 6$  integrins compared with normal cells has been demonstrated. The expression of  $\alpha 6 \beta 1$  integrin on prostate cancer cells was linked to increased invasion of prostate cancer in a mouse model (Schmelz et al., 2002). We and other groups have studied cell interactions with extracellular matrix and stromal factors during disease progression by characterizing integrin usage and expression in a series of parental and lineage-derived LNCaP human prostate cancer cell lines (Cress et al., 1995; Allen et al., 1998; Bello-DeOcampo et al., 2001; Edlund et al., 2001; Cooper et al., 2002; Schmelz et al., 2002). Although studies indicated the decrease of integrin heterodimers, the actual integrin expression on the cell surface showed no significant change; however, with disease progression, integrin usage did change significantly. The more metastatic sublines were distinct in their use of  $\alpha v \beta 3$  integrin (Edlund et al., 2001). When compared with parental LNCaP cells, the more metastatic sublines showed a shift in  $\alpha 6$  heterodimerization, a subunit critical not only for interaction with prostate basal lamina but also for interaction with bone matrix proteins, a favored site of prostate cancer metastases (Edlund et al., 2001). This indicates that integrin usage changed during the progression of prostate cancer. The activation state of integrins could be an important element in how cells adapt under different microenvironmental conditions. The adaptive property of integrins could be enhanced further



with changes in mediators or ECMs during the metastatic progression of prostate tumor epithelial cells, a potential step toward migratory properties.

The  $\alpha\text{v}\beta 3$  integrin heterodimer has been detected on many different cell types, such as macrophage, endothelial cells, osteoclasts, and cancer epithelial cells. The activation of  $\alpha\text{v}\beta 3$  in prostate cancer cells is mediated by the FAK pathway that activates the downstream PI-3 kinase/Akt pathway (Zheng et al., 2000). This triggers alterations in cell adhesion and migration on a variety of extracellular matrix proteins, including vitronectin, fibronectin, fibrinogen, laminin, collagen, and osteopontin.  $\alpha\text{v}\beta 3$  has been shown to be important for prostate cancer bony metastasis by adhesion of cancer cells to bone matrix components, vitronectin, osteopontin, and bone sialoprotein (BSP). Through analysis of DU145 cell adhesion to ECM, Zheng and colleagues showed that the adhesive property of DU145 cells can be decreased by LM609, a blocking  $\alpha\text{v}\beta 3$  antibody (Zheng et al., 1999; 2000). Osteopontin and vitronectin are common proteins in mature bone and can potentially serve as ligands for  $\alpha\text{v}\beta 3$ .

Integrin-associated protein (IAP/CD47) is a 50 kDa single-chain protein composed of an extracellular immunoglobulin superfamily (IgSF) domain, five membrane-spanning sequences and a short cytoplasmic tail. IAP was first isolated as a protein associated with the integrins,  $\alpha\text{v}\beta 3$ ,  $\alpha\text{IIb}\beta 3$ ,  $\alpha\text{v}\beta 5$ , and  $\alpha 2\beta 1$ . Human cells that lack IAP are deficient in  $\alpha\text{v}\beta 3$ -mediated ligand binding (Porter and Hogg, 1998). In an affinity purification study in which the 179–208 peptide of the  $\alpha 3(\text{IV})$  chain of collagen IV was used as the immobilized element, five proteins from melanoma and prostate cells were isolated. The 3 proteins were shown to be CD47/IAP, the integrin  $\beta 3$  subunit, and the  $\alpha\text{v}\beta 3$  integrin complex, respectively (Shahan et al., 1999), indicating that  $\alpha\text{v}\beta 3$  and IAP formed a complex in prostate cancer cells that could activate the functional property of  $\alpha\text{v}\beta 3$  in prostate cancer epithelial cells. Another recent study indicated that  $\alpha\text{v}\beta 3$  inhibits endothelial cell apoptosis during angiogenesis through NF- $\kappa\text{B}$  activation (Cooper et al., 2002). Angiogenesis facilitates the growth and metastasis of tumors by providing support and facilitating cancer migration. Together these studies suggest that  $\alpha\text{v}\beta 3$  is important not only for the growth and survival of tumor epithelium but for its supporting endothelium.

## Growth factors

The expression of *basic fibroblast growth factor* (bFGF, FGF-2) has been shown to be significantly increased in stromal fibroblasts in human prostate cancer and in endothelial cells compared with normal tissue. Prostate carcinoma cells have been shown to up-regulate fibroblast growth factor receptor isoforms with a high affinity for bFGF during cancer progression (Dow and de-

Vere White, 2000). Accordingly, elevated sensitivity to bFGF may stimulate cancer cell proliferation and protease expression, thereby supporting tumor growth and invasion. In addition, overexpression of both FGFR-1 and FGFR-2 in prostate cancer epithelial cells in a subset of prostate cancers has been correlated with poor differentiation. Thus, there is both an increase in bFGF concentration in stromal cells and increased expression of receptors in tumor epithelial cells which respond to bFGF, establishing a potential paracrine loop between prostate cancer cells and their surrounding stromal cells, which may be important for prostate cancer progression (Giri et al., 1999).

bFGF also stimulates fibroblast proliferation and extracellular matrix turnover through increased deposition and protease degradation (De Benedetti and Harris, 1999; Dow and deVere White, 2000), and functions as an angiogenic factor that induces endothelial cell migration, proliferation, and differentiation into new blood vessels (De Benedetti and Harris, 1999). Thus, bFGF may promote prostate cancer progression by inducing angiogenesis and stromal remodeling. A study also indicates the increase of bFGF in tumor epithelial cells due to induction of stromal FGF-2 (Giri et al., 1999), thus potentially establishing a positive feedback loop. Human prostate cancer cell lines DU-145 and PC-3 have been shown to express FGF-2 and metastasize to bone (Dow and deVere White, 2000). Furthermore, studies of the Dunning rat model show that activation of bFGF expression accompanied progression of epithelial cells to malignancy (Yan et al., 1993). These data suggest a possible contributing role for bFGF in the vicious cycle of tumor formation and progression.

*Platelet-derived growth factor* (PDGF) is a 30-kD protein consisting of disulfide-bonded homodimers or heterodimers of A and B subunits, also designated as c-sis (Sitaras et al., 1988). Its isoforms have been indicated as important during embryonic development, particularly in the formation of connective tissue in various organs (George, 2001). In adult tissues, the primary function of PDGF is to stimulate wound healing via chemotaxis and mitogenesis of fibroblasts, and secretion of extracellular matrix components (Tuxhorn et al., 2001). The normal physiologic targets for PDGF are stromal cells such as fibroblasts, endothelial cells, smooth muscle cells, and glial cells. Thus, paracrine release of PDGF stimulates stromal reactions in normal and pathologic states. Receptor binding by PDGF is known to activate intracellular tyrosine kinase, leading to autophosphorylation of the cytoplasmic domain of the receptor as well as phosphorylation of other intracellular substrates. This reaction is described as one in trans, i.e., the two receptor molecules of the receptor dimer phosphorylate each other. Specific substrates identified with the beta-receptor include Src, GTPase Activating Protein (GAP), phospholipase C (PLC) and phosphatidylinositol 3-phosphate. Both PLC- $\gamma$  and GAP seem to bind with different affin-



ities to the  $\alpha$ - and  $\beta$ -receptors, suggesting that the particular response of a cell depends on the type of receptor it expresses and the type of PDGF dimer to which it is exposed. In addition to the above, a non-tyrosine phosphorylation-associated signal transduction pathway can also be activated that involves the zinc finger protein Erg-1 (Khachigian and Collins, 1998).

Immunohistochemical analysis of PDGF and PDGFR indicated expression in both prostate epithelial and stromal cell types and in PIN lesions (Fudge et al., 1996). In contrast, the normal epithelial cells do not express PDGF nor PDGFR (Fudge et al., 1994). *In vitro* study of PDGF indicates that release of PDGF from tumor cell lines stimulates prostate stromal cell proliferation (Vlahos et al., 1993). This suggests that *de novo* expression of PDGF occurs early in prostate tumor progression. The production and activation of PDGF could further enhance the stromal reaction and contribute to the vicious cycle of tumor progression.

Recruitment of new blood vessel growth clearly illustrates the importance of tumor-stromal interactions during cancer progression. In normal human prostate tissue, VEGF is reportedly expressed at low levels and restricted to stromal cells. In high-grade PIN and prostate cancer, elevated expression of VEGF was observed in cancer, stroma and vascular endothelium (Ferrer et al., 1997). Endothelial cells from microvessels in the surrounding stroma must be induced to migrate into the tumor, whereby they proliferate and form new blood vessels to support tumor growth. This complex process is regulated by a delicate balance of angiogenesis inducers and angiogenesis inhibitors in the extracellular milieu. Increased activator(s) and/or decreased inhibitor(s) alter the balance and lead to the growth of new blood vessels (Hanahan, 1997). Several growth factors, such as VEGF, PDGF, TGF- $\beta$  and connective tissue growth factor, from epithelium or stroma, could induce angiogenesis (Nadal et al., 2002).

Recent studies indicate that VEGF directly stimulates prostate tumor cells via autocrine and/or paracrine mechanisms (Sokoloff and Chung, 1998; Jackson et al., 2002). One example demonstrated the possible role of VEGF as a mediator in the vicious cycle of tumor and stroma, in which reactive oxygen species (ROS) could participate in early prostate cancer epithelium growth and development. Increased ROS could enhance the production of VEGF, further promoting ROS concentration in stromal fibroblasts. The resulting overexpression of VEGF from stromal fibroblasts could induce Nox1, MMP-9, VEGF, and VEGFR production and increase the overall tumor growth rate (Arbiser et al., 2002).

In normal prostate tissue, IGF-1 is produced only by stromal cells, while prostate epithelial cells express insulin-like growth factor binding proteins (IGFBP-2, 3, 4, and 6) and the type I IGF receptor (Lopaczynski et al., 2001). It has been shown that both bFGF and PDGF can enhance IGF-1 production from endothelial cells.

Prostate cancer patients have shown increased serum IGF-1 and a decrease of IGFBP-3 level (Mantzoros et al., 1997; Chan et al., 1998; 2002; Chokkalingam et al., 2002; Grimberg et al., 2002). The increased serum IGF-1 concentration in prostate cancer patients could be from the stromal cells, metastatic prostate cancer epithelial cells or the liver. However, the study of IGF secretion is controversial. Some reports did not detect IGF-1 production by prostate cancer cells (Cohen et al., 1991; Pietrzkowski et al., 1993; Connolly and Rose, 1994; Angeloz-Nicoud and Binoux, 1995), others indicated the expression of IGF by prostate cancer cells (Iwamura et al., 1993; Kimura et al., 1996; Kaplan et al., 1999). Interactions between the glandular epithelium and the myofibroblasts and fibroblasts of the stromal compartment of the prostate gland appear to be regulated by IGF-1 availability. IGF-1 may act directly through the androgen receptor pathway and may be regulated through EGF-TGF- $\alpha$  receptor regulatory signaling (Kimura et al., 1996). This suggests the possible vicious cycle of IGF-1 production in prostate cancer progression. First, release of bFGF and PDGF induces IGF-1 secretion from prostate stromal cells, which could induce the increased production of androgen receptor and/or EGF from prostate tumor epithelial cells. The enhanced expression of the androgen receptor and elevated release of EGF can, in turn, further stimulate the release of IGF-1 from stromal cells, which may promote the progression of prostatic carcinoma cells.

Two huge molecules called *plasminogen-related growth factors (PRGFs)*, evolutionarily related to plasminogen, play an important role in inducing invasive growth of cancer progression. PRGF-1 is also called *hepatocyte growth factor/scatter factor (HGF/SF)*. PRGF-2 is also known as *macrophage-stimulating protein (MSP)*, *scatter factor-2* (Comoglio et al., 1999). HGF/SF has been demonstrated to be important in prostate cancer progression and metastasis, while MSP may be an important neurotrophic factor in embryonic development by inducing superoxide anion production (Brunelleschi et al., 2001; Rampino et al., 2002). It has been shown that both HGF/SF and MSP were up-regulated in the wound repair process in a rat model (Cowin et al., 2001). HGF/SF predominantly participates in a paracrine network. Several mesenchymal-derived cells (fibroblasts) secrete HGF/SF. It has been implicated as a mediator involved in communication between epithelial cells and the microenvironment (Comoglio and Trusolino, 2002). HGF/SF is secreted predominantly by stromal fibroblasts and stimulates proliferation and migration of epithelial and endothelial cells during organ development and tissue remodeling (Parr and Jiang, 2001). The secretion of HGF/SF as an inactive pro-HGF, which is converted into its bioactive form by a proteolytic cleavage by four proteases: urokinase (uPA), serine protease in the serum, coagulation factor XII, and its homologues (Comoglio et al., 1999). It has also been shown that some epithelial



cells secrete two potent inhibitors of pro-HGF activation (HAI-1 and -2) that tightly control HGF/SF activation (Denda et al., 2002).

In normal prostate epithelial cells, HGF/SF secreted by stromal cells causes growth inhibition, sustained phosphorylation of mitogen-activated protein kinase, and increased gene expression consistent with cell differentiation. Several soluble factors increase the production of HGF/SF in myofibroblasts but not in normal prostate epithelial cells, such as IL-1 $\beta$ , PDGF, bFGF, VEGF, and EGF (Zhu and Humphrey, 2000). Increased expression of the HGF/SF receptor c-Met proto-oncogene has been associated with progression of several types of carcinoma, including that of the prostate (Humphrey et al., 1995; Pisters et al., 1995; van Leenders et al., 2002). As mentioned above, the vicious cycle of bFGF, PDGF, and VEGF could further mediate or enhance the secretion of HGF/SF from stromal cells. Several studies have indicated that the increased concentration of HGF/SF in ECM could further contribute to malignancy in DU145 or PC-3 prostate tumor cells, inducing migration (Gmyrek et al., 2001; Nishimura et al., 1999).

## Cytokines

In a normal homeostatic state, IL-6 levels are typically very low. However, in response to microenvironment inflammatory factors, IL-6 can be released by wide variety of cell types. Cells known to express IL-6 include CD8+ T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells (under the influence of endothelins), neurons, neutrophils, monocytes, colonic epithelial cells, and B cells. IL-6 production is generally correlated with cell activation. Studies of tumor stroma indicate that increased IL-6 could induce the progression of prostate tumor epithelial cells by inducing the release of other cytokines, bone resorption, and induction of thrombopoiesis (Smith et al., 2001). Several groups have reported elevated serum levels of IL-6 upon progression of prostate cancer to androgen-independence (Nakashima et al., 2000; Shariat et al., 2001). It is possible that mediators released from prostate tumor epithelial cells could further enhance the production of IL-6 from both stromal and inflammatory cells. The increase concentration of IL-6 in ECM may further induce prostate tumor epithelial cells to produce mediators and IL-6R. Once prostate cancers reach malignancy, the tumor epithelial cells can produce IL-6 themselves and form an active autocrine loop.

A recent study indicated the increase of both IL-6 and IL-6R in prostate tumor epithelial cells with the increase of malignancy (Giri et al., 2001), again suggesting a "vicious cycle" mediated by IL-6 during the early development of prostate cancer and becoming an increasingly active autocrine loop in highly metastatic tumors. Similar vicious cycles have been shown for other cytokines,

such IL-8. In human prostate cancer, IL-8 has been shown to stimulate PC-3 prostate cancer cell migration and invasion *in vitro* through a reconstituted basement membrane and both long-term migration and short-term adhesion to laminin (Reiland et al., 1999). IL-8 is produced by many different stromal cells, including endothelial cells. IL-8 is also produced by various metastatic tumor cells, including prostate cancer cells (Kim et al., 2001). Furthermore, stress factors, such as hypoxia, acidosis, nitric oxide (NO), and cell density, which increase with the progression to malignancy, can also influence IL-8 production (Shi et al., 2000).

## Stromal response to early prostate inflammatory atrophy

Inflammatory reactions often result in the activation and recruitment of phagocytic cells (e.g., neutrophils and/or tissue macrophages) whose products, such as cytokines, oxidants and free radicals, result in injury to the tissue. Recent reports indicate that benign prostatic hyperplasia (BPH) frequently exhibits infiltration of CD4(+)/CD45RO(+) memory T-lymphocytes. This infiltration could induce growth of myofibroblast cells in BPH. Increased level of cytokines, such as IL-2, IL-4 and TNF, were also detected in T-cells in BPH but not in normal prostate (Kramer et al., 2002). Another study also indicated the association of inflammation with BPH and prostate cancer, and the increased expression of Bcl-2 in these prostate patients (Gerstenbluth et al., 2002). The chronic inflammation status linked to the development of tumor has been reported in several organ systems, including prostate cancer (De Marzo et al., 1999). The hypothetical mechanism involves repeated tissue damage and regeneration in the presence of highly reactive oxygen. These reactive molecules, i.e. hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) released from the inflammatory cells interact with DNA in the proliferating epithelium to produce permanent genomic alteration, such as frame-shift mutation, deletions, and rearrangements, as well as increasing the epithelial proliferative rate (Gasche et al., 2001; Oda et al., 2001). Recent studies of prostate cancer indicated inflammatory responsive cells at the juxtaposition of highly proliferative prostate epithelial cells, referred to as a Proliferative Inflammatory Atrophy lesion (PIA lesion). Studies demonstrated mononuclear and/or polymorphonuclear inflammatory cells in both the epithelial and stromal compartments, and stromal atrophy with variable amounts of fibrosis. Luminal epithelial cells of PIA lesions have elevated levels of Bcl-2, decreased expression of p27<sup>Kip1</sup>, and increased levels of  $\pi$ -class glutathione S-transferase (GSTP1) (De Marzo et al., 1999). *In vivo* study of H<sub>2</sub>O<sub>2</sub> and GSTs indicated that H<sub>2</sub>O<sub>2</sub> enhances the expression of GSTP1 (Liu et al., 2001). This indicates that increase expression of GSTs in PIA may be due to increased concentrations of H<sub>2</sub>O<sub>2</sub> in the stromal microenvironment during



the PIA stage. However, PIN and prostate cancer cells rarely express GST $\alpha$  isoenzyme (GSTA1) and GSTP1 as a result of increased methylation of GSTP1 in a “CpG island” which inactivates GSTP1 (De Marzo et al., 1999; Parsons et al., 2001).

The phenotypic switch of stromal cells, extracellular matrix remodeling, increased growth factor availability, elevated protease activity, increased angiogenesis, and recruitment of inflammatory cells were observed in cancer progression. The stromal response to cancer shows similarity to the wound repair response (Tuxhorn et al., 2001), and it is possible that these conditions could promote tumorigenesis. The phenotypic switch between fibroblast and myofibroblast indicates increased extracellular matrix remodeling during prostate cancer progression. In normal prostate it has been reported that the “stromal network of collagen fibers is loosely woven, fine and smooth in texture,” while in Gleason-score seven adenocarcinoma the collagen fibers “appeared swollen in diameter” and there was “no regularity in the spatial relationship of the fibers” (Keller et al., 2001; Tuxhorn et al., 2001). This suggests that remodeling of the extracellular matrix is one of the key features of stromal reaction in prostate cancer.

## Prostate cancer and bone stromal interaction

The progression of prostate cancer from the androgen-dependent to the androgen-independent and bone metastatic state is considered a poor and generally lethal prognosis. To understand the molecular basis of disease progression and develop rational new therapeutic approaches for targeting prostate cancer bone metastasis, we must first understand the multi-step processes that lead to prostate cancer metastasis to bone. As depicted in Fig. 2, at the site of primary cell growth we expect prostate cancer cells to interact with prostate stromal cells and gain the ability to extravasate into the bloodstream. In the blood, prostate cancer cells are expected to survive and move as an embolus prior to adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. At the final step of this progression, prostate cancer cells interact directly with osteoblasts and osteoclasts through a series of soluble factors (e.g., receptor activator of NF- $\kappa$ B ligand, RANKL) via cell surface receptor (e.g., RANK) to survive, proliferate, migrate and invade and eventually replace the bone marrow components. To understand the cellular and molecular basis of the prostate tumor-bone stroma interaction, it is essential to delineate how the soluble growth factors and extracellular matrices participate reciprocally in the progression of prostate cancer toward androgen-independence and bone metastasis.

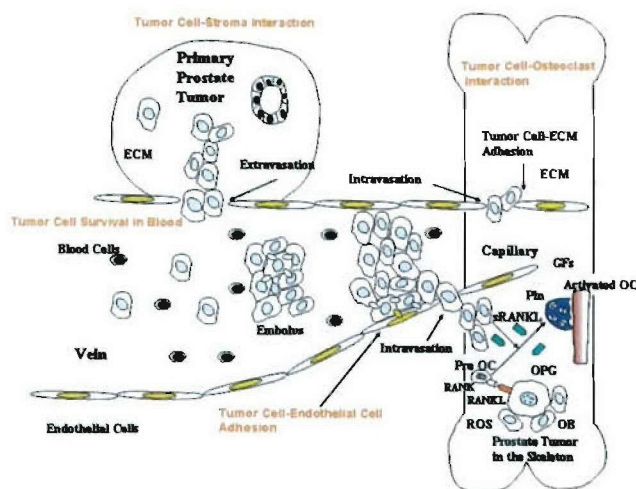


Fig. 2 The multi-step processes of prostate cancer metastasis to bone. Once prostate cancer cells gain the ability to extravasate into the bloodstream, prostate cancer cells move as embolus prior to adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. Prostate cancer cells then interact directly with osteoblast and osteoclast through a series of soluble factors via cell surface receptor (e.g., RANK) to invade and replace the bone marrow components.

A conceptual framework will be introduced here to illustrate the following issues. 1) Bone stromal reaction to cancer epithelium may signal further tumor progression. Altered bone stromal cells, in response to tumor epithelium, may induce further epithelial genetic and phenotypic changes and thus contribute to a vicious-cycle cascade in the androgen-independent and metastatic progression of prostate cancer (Fig. 1). 2) Co-targeting tumor and stroma could starve or kill tumor cells from their supporting microenvironment and could offer the greatest benefits for inducing tumor regression and sustaining the long-term survival of patients with prostate cancer skeletal metastasis and its associated complications.

By using a human prostate cancer co-culture model, our laboratory has obtained evidence suggesting that non-random genetic changes occur in human bone stromal cell line MG-63, after co-culturing with the human androgen-independent prostate cancer cell line C4-2 (a lineage-derived LNCaP subline with growth and metastatic potential to lymph node and bone when injected subcutaneously or orthotopically in castrated mice) under 3-D conditions. The 3-D model is valuable for the evaluation of the prostatic tumor-bone stroma interaction in vitro. The participation of bone stroma in tumor growth and progression suggests that when prostate cancer metastasizes to bone, there are complex and reciprocal cellular interactions between populations of tumor and host bone cells.



## **“Vicious cycle” between prostate cancer and bone stroma**

### Laboratory observations

While clinical human prostate cancer is predominantly osteoblastic, the established human prostate cancer cell lines inoculated and grown in the bone of immune-compromised mice yield both osteoblastic and osteolytic lesions. Apparently, prostate cancer cells can participate in the process of bone turnover by exhibiting properties similar to osteoblasts, the so-called “osteomimetic” properties of prostate cancer cells as reported earlier (Koenen et al., 1999). Much evidence supports this interesting phenotype of prostate cancer cells, in which they behave like osteoblasts. Prostate cancer cells express both soluble and membrane-bound RANK ligands and were shown to participate directly in osteoclastogenesis (Koenen et al., 1999; Matsubara et al., 2001; Zhang et al., 2001; Yeung et al., 2002). Prostate cancer cells expressed a number of non-collagenous bone matrix proteins, such as osteocalcin, osteopontin, osteonectin and bone sialoprotein, alkaline phosphatase, and a key transcription factor, Runx 2 (cbfa1) that controls the transcription of osteocalcin and collagenous-3 (D’Alonzo et al., 2002). In addition, upon exposure to mineralizing cell culture conditions, prostate cancer cells have been shown to form *bona fide* mineralized bone crystals as detected by electron microscopy (Lin et al., 2001). These observations raise the possibility that soluble and/or matrix-associated molecules may be responsible for signaling between prostate cancer and bone stromal cells. Since bone-homing prostate cancer cells seek to adhere, colonize, and survive in bone, it is of pivotal importance to find out how prostate tumor and bone cells interact with the hope of identifying novel therapeutic targets for the treatment of prostate cancer bone metastasis. One attractive hypothesis is that prostate cancer cells may behave like osteoblasts and functionally participate in bone turnover. By markedly increasing the basal rate of bone turnover, this may further enhance prostate cancer cell colonization in bone (Cher, 2001; Nemeth et al., 2002). This hypothesis is supported by some clinical observations, where bisphosphonates, an effective class of agents that slow down or inhibit bone resorption, have been shown to reduce cancer cell colonization in experimental models of prostate and breast cancers (Coleman, 2001; Lee et al., 2001). In men harboring prostate cancer, there is evidence that increased bone resorption occurs upon castration. Whether these changes in bone turnover subsequent to hormonal manipulation or bisphosphonate treatment after prostate cancer cell colonization in bone affect the natural history of prostate cancer progression should be the subject of future thorough investigation.

Factors driving the “vicious cycle” between prostate cancer and bone cells

Guisse and colleagues (Chirgwin and Guise, 2000) and Mundy (Mundy, 2002) presented the concept of a “vicious cycle” involving TGF- $\beta$  produced by bone cells that promotes the production of PTHrP by many of tumor cells, including prostate and breast tumor cells. PTHrP stimulates bone turnover by enhancing osteolytic reaction in the bone. Increased release of TGF- $\beta$  could result from rapid bone turnover, and this may trigger increased PTHrP production by cancer cells. The production of PTHrP by tumor cells will induce osteolytic cells to express an increased level of RANK ligands, which can promote osteoclast formation/activation and subsequently increased bone resorption. The enhanced resorptive process by osteoblasts and osteoclasts leads to “bone pitting” and subsequent colonization by cancer cells in the skeleton and associated bone destruction often observed in cancer patients. Thus, a “vicious cycle” may exist between TGF- $\beta$ , PTHrP, RANK ligands in osteolytic prostate cancer. Interrupting the vicious cycle in cancer models using anti-PTHrP antibodies or osteoprotegerin (OPG) has been shown to reduce colonization of cancer metastasis to bone (Zhang et al., 2001).

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a 25-kDa disulfide-linked polypeptide which coordinates cell function over distances by binding to cell surface receptors. An immunohistochemical study of mouse prostate development indicated that TGF- $\beta$ 1 is expressed in mesenchymal cells (Timme et al., 1995). It was initially characterized by its effects on epithelial function and proliferation (Cui et al., 1995), but it is also an important mediator of stromal reaction (Wakefield and Roberts, 2002). Responses to TGF- $\beta$  include phenotypic changes affecting adhesion, migration, differentiation, and cell fate. In general, TGF- $\beta$  stimulates the production of ECM components, inhibits degradation, and alters integrin expression. It follows that all of these effects can significantly alter cell behavior.

TGF- $\beta$  is abundant in latent forms that circulate or are bound to the ECM in bone. Activated TGF- $\beta$  can bind to ubiquitous heterodimeric receptors and induce signal cascade through the SMAD pathway (Taipale et al., 1998). Stromal and epithelial cells of malignant and nonmalignant prostatic tumors express all three TGF- $\beta$  isoforms and their related receptors which act as paracrine and autocrine factors, influencing prostate function and stromal-epithelial cell interaction (Cardillo et al., 2000). These data indicate that TGF- $\beta$ 1 produced by carcinoma cells acts on the surrounding stromal cells, which in turn induces stromal cells to release cytokines to further promote the malignancy of the cancer cells.

In addition to the TGF- $\beta$  and PTHrP connection, a number of other candidate molecules may also contribute to the vicious cycle of cancer growth and bone meta-



stasis. For example, bone is a rich source of hydrogen peroxide, and hydrogen peroxide has been shown to increase the production of vascular endothelial growth factor (VEGF) by tumor cells. There is evidence that increased VEGF could further stimulate increased production of hydrogen peroxide by tumor and bone cells. Since VEGF is known to be required to support tumor growth and colonization, it is possible that a hydrogen peroxide/VEGF connection contributes to the vicious cycle between tumor and bone cells (Arbiser et al., 2002).

Endothelin-1 (ET-1) may also contribute to osteoblastic reaction when prostate cancer cells colonize to bone. ET-1 production is negatively regulated by androgen. Thus castration could potentially reduce osteoblastic reactions in bone through the reduction of ET-1. However, ET-1 and its interaction with receptor ET-1A could participate in the osteoblastic reaction and spur the vicious cycle in prostate cancer and bone by increased production of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , and TGF- $\beta$  (Le Brun et al., 1999; Granchi et al., 2001). Increased production of TGF- $\beta$ , EGF and IL-1 $\beta$  has been shown to upregulate ET-1, hence altering the growth factor and cytokine milieu in bone in response to ET-1 growth factor (Granchi et al., 2001). Cytokines may contribute to further prostate cancer growth and colonization to bone.

ET-1, composed of 21 amino acid residues, was originally isolated from porcine aortic endothelial cells (Kurihara et al., 1989). ET-1 is one of the four families of vasoactive peptides that include endothelin-2 (ET-2), endothelin-3 (ET-3), and endothelin-4 (ET-4) (Cunningham et al., 1997). All members of the endothelin family contain two essential disulfide bridges and six conserved amino acid residues at the C-terminus. In addition, they all are synthesized as pre-pro-polypeptides which need to be cleaved to produce pro-polypeptides. The pro-ET-1 is proteolytically cleaved by a membrane-bound metalloproteinase, endothelin-converting enzyme (ECE-1), produced by endothelial and epithelial cells (Xu et al., 1994). Two receptors for endothelins have been characterized, designated ETA and ETB. Although these receptors are structurally and functionally different, they share some similarities. Both are seven membrane domain receptors coupled through G proteins to phospholipase C. Both have an N-terminal signal sequence and a long N-terminal extracellular domain (Sakurai et al., 1992). ETA shows a higher affinity for ET-1 than for ET-2 and the lowest affinity for ET-3. The ETB receptor shows approximately equal affinity for each of the endothelins. Both ETA and ETB have been identified in prostate tissue. Stroma has higher concentration of ETA, while ETB is predominately in the epithelial cells of the prostate (Remuzzi and Benigni, 1993).

In human prostate cancer progression, ET-1 and ETA expression is retained, whereas ETB receptor expression is reduced. ET-1 protein expression was detected *in situ*

in 14 of 14 primary cancers and 14 of 16 metastatic sites. Exogenous ET-1 induces prostate cancer proliferation directly and enhances the mitogenic effects of IGF I, IGF II, PDGF, bFGF, and EGF in serum-free conditions *in vitro*. ETA antagonist A-127722 inhibits ET-1-stimulated growth, but the ETB-selective receptor antagonist BQ-788 does not. ET-3, an ETB-selective agonist, also had no effect on prostate cancer growth. No specific ETB-binding sites could be demonstrated in any established human prostate cancer cell line tested, and ETB mRNA, detected by reverse transcription PCR, was reduced. The predominance of ETB binding in human benign prostatic epithelial tissue is not found in metastatic prostate cancer by autoradiography. Furthermore, a study of ET-1 in prostate cancer bone metastasis demonstrated that ET-1 is mitogenic for osteoblasts, inhibits osteoclastic bone-resorption, and induces the formation of osteoblastic lesions. All this suggests that ET-1 is involved in the new bone formation associated with prostate cancer metastasis (Nelson et al., 1999).

Tests of the mitogenic property of ET-1 indicated that other factors also can be co-factors with ET-1, such as bFGF and IGFs. PDGF and ET-1 also can play a role in tumor angiogenesis in conjunction with VEGF. Clinical trials of ET-A receptor antagonist in prostate cancer indicated that it could help patients, if they could tolerate mild but pervasive symptoms related to ET-1's vasoconstrictive effects (Kopetz et al., 2002).

A recent study (Taichman et al., 2002) showed that stromal chemokine and receptor, such as stromal cell-derived factor-1 (SDF-1 or CXCL12) and its receptor (CXCR4), may play a role as prostate cancer bone metastasis homing signals. The level of CXCR4 increased with the malignancy of the prostate cancer cell lines by both RT-PCR and Western blot analysis. The increased expression of CXCR4 also increased spreading to bone in animal studies. An *in vitro* study of cellular spreading in basement membrane indicates that spreading can be inhibited by CXCR4 antibody. These findings suggest that chemokine and its receptor could also be important in prostate cancer bone metastasis.

Together, these studies indicate that the process of prostate cancer bone metastasis is a complicated pathway requiring multiple chemokines, cytokines, and membrane proteins. These complexities also suggest the possibility of therapeutic strategies specifically focused on co-targeting and disrupting key carcinoma-stroma interactions.

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## Cancer therapy based upon co-targeting tumor and stroma

### Laboratory and clinical observations

Because prostate cancer growth is highly susceptible to tumor-microenvironment interaction and experimen-



tally can be promoted by stromal fibroblasts, it is reasonable that control of prostate tumor growth might be optimized by co-targeting both tumor and stroma. To explore this concept, we designed studies to co-culture prostate cancer cells and bone stroma *in vitro*, establishing chimeric tumor models consisting of human prostate cancer cells and bone stroma. By introducing a “bystander” therapeutic gene, herpes simplex thymidine kinase (hsv-TK), to stromal cells only, we observed effective cell kill in tumor epithelium *in vitro* and shrinkage of tumor size *in vivo* upon addition of a pro-drug, gancyclovir (GCV). Since there were no identifiable gap junctions between prostate tumor cells and bone stroma under the electron microscope, we concluded that there must be metabolic cooperation between tumor epithelium and bone stroma mediated by soluble factors and extracellular matrices. By interrupting this communication, and targeting both tumor and stroma, tumor growth and survival may be adversely affected. Conceptually, co-targeting tumor and stroma in prostate cancer bone metastasis is a rational approach to the “vicious cycle” constantly operating between tumor and stroma. Directly inducing cell-kill of tumor epithelium and starving cancer cells by disrupting tumor interaction with the stromal compartment could achieve the best possible tumor regression.

In our laboratory, we co-targeted tumor and stroma using an adenoviral vector in which therapeutic gene expression was controlled by a tissue-specific and tumor restrictive promoter, such as osteocalcin, osteonectin, or bone sialoprotein. These have been shown to be highly effective in inducing long-term tumor regression, and even some cure in pre-established tumor in the skeleton with administration of the adenovirus through the intravenous route (Hsieh and Chung, 2001; Matsubara et al., 2001; Hsieh et al., 2002). This concept of bone targeting to improve therapeutic effects has received clinical support. Tu and colleagues (Tu et al., 2001) reported a significant prolongation of patient survival by targeting bone with strontium 89 and prostate tumors with chemotherapy.

### Molecular basis of co-targeting

The bone microenvironment was depicted by Paget over a century ago as a specialized “soil” that favors the metastasis of certain selective cancer cell types (“seed”). While the precise mechanism by which cancer cells home to bone is still unknown, several attractive ideas and hypotheses have been proposed. Bone must express certain chemo-attractants that selectively retain circulating cancer cells, and cancer cells must express cognate ligands or receptors allowing them to attach to bone marrow-associated endothelial cells, marrow stromal cells or osteoblasts, and/or respond to bone-derived growth factors, cytokines/chemokines or extracellular

matrices. To metastasize to bone, cancer cells must be able to survive “hostile” circulatory compartments, including the blood and lymphatic channels. The mere detection of cancer cells in blood or marrow stromal compartments may not reflect the “vitality” of cancer cells. Solakoglu, et al (Solakoglu et al., 2002) recently demonstrated that the outgrowth of cytokeratin-positive tumor cells from bone marrow can be detected in 81% of prostate cancer patients. Increased cell viability in patients correlated with increased cancer-related deaths.

From our use of prostate cancer cell lines as a model to study carcinoma-stroma interaction, we suggest that a switch of transcriptional factors must occur during the pathogenesis of prostate cancer. This biochemical switch could occur early, even when epithelial cells are still in the primary stage, since even then the expression of bone-like proteins such as osteocalcin, osteopontin, osteonectin, and bone sialoprotein was detected. Considering how osteocalcin promoter in prostate cancer cells is regulated, a vicious cycle could occur at the level of transcription factor activation, wherein the coordinated activation of transcription factors by known soluble factors and ECM-integrin signaling culminates in the ability of prostate cancer cells to proliferate and survive in bone. Numerous links have been established between the up-regulation of transcription factors such as Runx-2 and the potential alteration of cellular behavior that could lead to increased cell growth and spread to bone.

Runx-2 is a potent and specific transcription factor that controls mesenchymal-epithelial interaction in tooth development (D’Souza et al., 1999). Apparently, Runx-2 activation is controlled by soluble growth factors, and upon activation it can regulate soluble growth factor secretion, which ultimately controls the growth and differentiation of enamel tooth epithelium. Based upon this and other published data, we proposed that activation of similar transcription factors such as Runx-2 in prostate cancer cells could potentially enhance prostate cancer cell invasion and migration through the induction of collagenase (e.g., collagenase 3) and other metalloproteinases. The concomitant induction of Runx-2, collagenase 3, and other growth and differentiation supportive factors could enhance prostate cancer survival and invasion. Similarly, the activation of the  $\alpha V\beta 3$  and  $\alpha 2V\beta 1$  integrin-ECM pathways may promote outside-in signals that result in enhanced cell migration and invasion.

We proposed earlier that prostate cancer metastasis to bone is not a random process. It involves the specific recognition of cancer cells by bone as “self” and the production of bone-like proteins by cancer cells. The expression of bone-like proteins by prostate cancer cells may allow them to adhere, proliferate and survive in the bone microenvironment and participate in certain normal functions of bone cells, i.e., bone resorption.

Cancer cells express a bone-like phenotype early, when they are in primary lesions. This raises the possi-



bility that the expression of bone-like proteins by cancer cells and reactive stroma may serve as a prognostic biomarker for prostate cancer bone metastasis and possibly as a predictor for patient survival. By combining the expression of bone-like proteins and the stromal reaction to epithelium, it is possible that novel molecular markers can be developed both at the gene expression and genetic level. While the role of bone-like protein is presently unclear, it is possible that the activation of these processes may occur at the transcription level. Transcriptional factor switching could be of fundamental importance in determining the phenotype of cancer cells and might influence the extent of the vicious cycle between tumor cells and bone stroma. It is possible that specific targeting of transcriptional factors could have benefit as cancer therapy. Interrupting the activation of bone-like proteins in tumor epithelium and bone stroma may prevent prostate cancer cell adherence, proliferation, and survival in bone.

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## Review Articles

# MOLECULAR INSIGHTS INTO PROSTATE CANCER PROGRESSION: THE MISSING LINK OF TUMOR MICROENVIRONMENT

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### ABSTRACT

**Purpose:** Tumor cell genotype and phenotype have been considered the only determinants supporting cancer growth and metastasis. This review focuses on the published literature that suggests that tumor-microenvironment interaction has a decisive role in controlling local cancer growth, invasion and distant metastasis. As this review shows, genetic alterations in prostate cancer cells alone are not enough to confer metastatic status without a supporting tumor microenvironment. Effective therapeutic targeting requires a deeper understanding of the interplay between tumor and stroma. Approaches co-targeting tumor and stroma already show promise over the conventional targeting of tumor cells alone in preventing prostate cancer progression and eradicating preexisting or newly developed prostate cancers in bone and visceral organs.

**Materials and Methods:** A literature survey using the MEDLINE database was performed in basic and clinical publications relevant to tumor-host microenvironment interaction. Information pertinent to the biology and therapy of prostate cancer local growth and distant metastases was specifically emphasized.

**Results:** Tumor associated stroma actively fuel the progression of prostate cancer from localized growth to the invasion of surrounding tissues, and the development of distant bone and visceral organ metastasis. In concert with this progression tumor cells recovered from metastatic sites could represent a subpopulation of preexisting tumor cells or could be a newly acquired variant subsequent to tumor-stromal interaction. Experimental data from our laboratory and others suggest that permanent genetic and phenotypic changes occur in prostate cancer cells after 3-dimensional co-culture in vitro or when co-inoculated and grown with inductive stromal cells in vivo. These results support the idea that newly acquired variants are the dominant mechanism of prostate cancer progression. Intercellular communication between prostate cancer cells and organ specific stroma, including prostate and marrow stroma, could involve diffusible soluble and solid matrix molecules as mediators, leading to the development of metastasis. This presents a new opportunity for therapeutic targeting for the treatment of benign and malignant growth of the prostate glands. This review summarizes specific research implicating tumor-microenvironment interaction as the molecular basis of cancer progression, providing a rationale for targeting tumor and the tumor associated microenvironment in the management of androgen independent and bone metastatic prostate cancer progression in patients.

**Conclusions:** Cancer is not a single cell disease. Aberrant cancer cells and their interactive microenvironment are needed for prostate cancer to progress to androgen independence and distant metastasis. It is highly plausible that newly evolved prostate cancer cell clones dominate cancer metastasis after cell-cell and cell-matrix interaction with the host microenvironment, rather than the selection or expansion of a preexisting prostate cancer cell clone(s). Based on this premise potential molecular targets in the microenvironment are especially emphasized. Further elucidation of the molecular mechanisms underlying tumor-stromal interaction may yield improved medical treatments for prostate cancer growth and metastasis.

**KEY WORDS:** prostate, prostatic neoplasms, neoplasm metastasis, disease progression, growth substances

Cancer cells reside in an organotypic host microenvironment that has long been underemphasized because it is perceived only as a silent bystander. As originally proposed in 1889 by Paget,<sup>1</sup> past understandings of the organ specific

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profile of cancer and metastasis (soil and seed) led to the idea that preexisting subpopulations of cancer cells successfully complete a rather inefficient process called metastasis.<sup>2,3</sup> Strong experimental evidence suggests that primary tumors are heterogeneous and subsequently observed metastasis is the result of a nonrandom, sequential, multistep selective process among preexisting cell subtypes.<sup>2,4</sup> Kauffman et al reviewed the roles of metastatic suppressor genes, of which



the loss of suppressor genes may prompt the selective growth and survival of cancer cells at certain secondary sites.<sup>5</sup> Chambers et al suggested that molecular interaction between cancer cells and their metastatic organ site determines the success of cancer colonization.<sup>6</sup> These selective processes are generally believed to occur rarely and during the late stages of tumor progression.<sup>3,5</sup> Hence, this raises the question of whether molecular profiling of cancer signatures at the primary prior to cancer metastasis can reliably predict the clinical outcome.<sup>7,8</sup> A compromise idea was proposed by Kang et al, who suggested that the expression of certain genes in primary breast cancer may indeed be prognostic but organ specific tropism can be achieved only after cancer cells have expressed a concrete set of overt bone metastasis genes.<sup>9</sup> In this understanding tumor microenvironment is the missing link that not only provides fertile soil for cancer growth, but also exerts dominant inductive influences that trigger permanent genetic and phenotypic changes in cancer cells, conferring their selective growth and survival advantages in subsequent dissemination. Thus, the characteristic metastatic cell clones to bone could have a loss of expression of metastatic suppressor genes, enhanced ability to interact with primary and secondary organ sites and acquisition of expression of a set of bone metastatic genes after cancer cells interact with the host microenvironment, at the primary or at metastatic sites.

The host microenvironment could participate actively in this rather inefficient and nonrandom metastatic process, in which cancer cell variants evolve after tumor-stromal interaction at primary or secondary sites of tumor growth. The molecular processes associated with this interaction are reviewed in several contexts. 1) There is a reciprocal cancer cell-microenvironment interaction that facilitates the development of osteomimicry and vasculogenic mimicry by cancer cells.<sup>10</sup> In other words, cancer cells can mimic the gene expression profiles of cells in their microenvironment. For instance, prostate cancer cells can express bone cell-like (osteomimetic) properties<sup>10,11</sup> and melanoma can express vascular endothelial specific markers in vasculogenic mimicry.<sup>12,13</sup> 2) The ability of cancer cells to undergo morphological transitions, such as epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition, is accompanied by the ability of cancer cells to acquire altered behaviors. Cancer cells observed to undergo EMT, often at the invasion front of a tumor, acquire increased motility, leading to migration and invasion.<sup>14-16</sup> 3) There are coordinated and stable changes in gene expression profiles noted in cancer cells grown as 3-dimensional (3-D) organoids with proliferation and survival intimately linked to the surrounding interface tissue matrix microenvironment.<sup>17-19</sup> Perturbations in cancer-matrix interaction have been observed to alter important cell functions, such as cell polarity, secretion, migration and invasion. Understanding the molecular mechanisms involved in the evolution of cancer cells, and the reversibility and vulnerability of this process could help in the design of future therapeutic agents. This review specifically considers the recent development of clinical trials targeting the tumor microenvironment, such as bone stroma, tumor angiogenesis, paracrine signaling, host immune directed and other co-targeting strategies for the treatment of prostate cancer and its metastasis.

#### EXPERIMENTAL MODELS DEMONSTRATE THAT TUMOR MICROENVIRONMENT SELECTIVELY ENHANCES CANCER PROGRESSION

There are 3 commonly used methods to enhance the tumorigenic and metastatic potential of human prostate cancer cells. They are to 1) transfect stably genetic materials to prostate cells that encode oncogenes, metastasis associated genes, inactivating tumor suppressors, cell cycle regulators and downstream mediators regulating cell proliferation, sur-

vival and apoptosis, 2) deprive prostate cancer cells of androgen in cultured medium or grow prostate tumors in surgically castrated hosts and 3) co-inoculate prostate cancer cells with relevant organ specific stromal cells or tumor derived extracellular matrices.<sup>20-22</sup> Of these 3 approaches androgen deprivation and tumor-stromal interaction seem to mimic best the natural history of prostate cancer progression in patients, ie the resulting tumor cells have the ability to undergo androgen independent and bone metastatic progression. Unfortunately the interplay between cancer cells and host stroma remains unclear because there is no transgenic animal model that specifically harbors a prostate cancer microenvironment defect, leaving us with a knowledge gap in defining the precise role of tumor microenvironment in cancer progression and metastasis. Nevertheless, from the published data we can conclude that the genetic make-up of a cancer cell and its host interaction shape the tumorigenic and metastatic potential of cancer cells. Since cancer growth and subsequent metastasis *in vivo* are possible only if experiments are performed in living animals, it is an inescapable conclusion that the host microenvironment must be involved even when genetically defined cells are tested.

Consistent with the described theme, it has been shown that orthotopic rather than ectopic inoculated prostate tumor cells acquire soft tissue and bone metastatic capability.<sup>23,24</sup> Permanently altered prostate cancer cell clones (phenotypic and genotypic) isolated after *in vivo* cellular interaction with bone stromal cells<sup>25</sup> or *in vitro* 3-D cellular interaction with prostate or bone derived stromal cells acquired bone and soft tissue metastatic potential.<sup>26,27</sup> These results imply that an organ specific stroma milieu comprised of different cell types that secrete different growth factors, extracellular matrices, metalloproteinases and/or angiogenic molecules must be responsible for the process driving nontumorigenic or nonmetastatic prostate cancer cells to yield tumorigenic and metastatic phenotypes. Direct evidence that factors from host rather than tumor contribute to angiogenesis and tumor formation was provided by Huang et al, who reported that human ovarian cancer growth as peritoneal tumors and ascites was lower in transgenic immune compromised nude mice lacking the MMP-9 gene due to the decreased level of extracellular matrix ECM remodeling and angiogenesis adjacent to the sites of tumor colonization.<sup>28</sup> They identified macrophages as the source of the MMP-9 supporting ovarian tumor growth, angiogenesis and spread. When they resolved the MMP-9 deficiency by transferring spleen cells from wild-type mice as a source of macrophages to MMP-9 deficient mice, the growth, angiogenesis and colonization of ovarian tumors in the recipient mice were restored.

Results such as these support the exciting concept that stroma is a potential target for ovarian cancer treatment.<sup>29</sup> Table 1 lists a number of well characterized, human prostate cancer, lineage related progression and xenograft models that could be used to study tumor-host microenvironment interaction. Among these models are the LNCaP progression model and the invasive ARCaP model established at our laboratory.<sup>24,25</sup> These models share important common features. They express 2 lethal phenotypes of human prostate cancer, androgen independence and bone metastasis, they consist of lineage related cell lines evolved from the parental cell clone with common genetic backgrounds but diverse phenotypes and behaviors, cells derived from each model express androgen receptor (AR) and secrete prostate specific antigen, and they were derived from *in vivo* tumors grown as 3-D xenografts under the influence of host factors and subjected to tumor-stromal interaction. The most remarkable aspect of these models is that the prostate cancer cells acquired increased tumorigenic and metastatic potential merely through cellular interaction with the host microenvironment under 3-D conditions without requiring the transfer of any exogenous genes to the indolent appearing cancer



TABLE 1. Human prostate cancer cell and xenograft models used to study androgen independent and metastatic progression of this disease

	Commonly Used Cell Lines	Comments	References
<b>Lineage related progression cell model:</b>			
LNCaP	C4, C4-2, C4-2B	Derived from LNCaP with increasing androgen independent + bone metastatic potential	Thalmann et al <sup>25, 69</sup>
ARCaP	ARCaP sublines	Single cell cloned or selected after metastasis to bone	Zhou et al <sup>24</sup> + Xu et al, Urology, suppl., 169: 81, 2003
PC-3	PC-3M, PC-3M-Pro4, PC-3M-LN4	Selected <i>in vivo</i> after orthotopic implantation of PC-3 cells	Stephenson <sup>23</sup> + Pettaway et al: Clin Cancer Res, 2: 1627, 1996
CWR22	CWR <sub>22</sub> R <sub>v1</sub>	Derived from hormone relapsed + CWR22 tumor grown in castrated host	Wainstein et al: Cancer Res, 54: 6049, 1994 + Sramkowski et al: In Vitro, 35: 403, 1999
RWPE-1	N-methyl-N-nitrourea (MNU) treated sublines	Exposure of immortalized human prostate epithelial cell line to MNU + developed sublines with increased tumorigenicity	Bello-DeOcampo et al: Mut Res, 480/481: 209, 2001
<b>Xenograft model:</b>			
MDA-PCa	MDA-PCa2a, MDA-PCa2b	Derived from bone metastatic specimens of single pt	Navone et al: Clin Cancer Res, 3: 2493, 1997
LuCaP	LuCaP <sub>35</sub> , LuCaP <sub>35v</sub> , LuCaP <sub>23-1</sub> , LuCaP <sub>23-12</sub>	Developed from pts with prostate Ca metastasis to lymph node + liver	Ellis et al: Clin Cancer Res, 2: 1039, 1996 + Corey et al: Prostate, 55: 239, 2003
LAPC	LAPC-8, LAPC-4, LAPC-9	Derived by implanting surgical specimens from pts into SCID mice	Klein et al: Nat Med, 3: 402, 1997
PC	PC-82, PC-133, PC-135, PC-295, PCEW	PC xenografts were established by growing primary prostate cancer or lymph node metastasis in nude mice	van Weerden et al: Prostate, 43: 263, 2000
DuCaP	DuCaP	Single cell line derived from dura mater of pt with prostate Ca	Lee et al: In Vivo, 15: 157, 2001
VCaP	VCaP	Single cell line derived from vertebra of prostate Ca metastasis	Korenchuk et al: In Vivo, 15: 163, 2001

cell lines. Control studies using similar cell types grown alone or with organ specific stromal cells under 2-D conditions failed to generate invasive and metastatic variants.

#### INTERCELLULAR COMMUNICATION BETWEEN CANCER CELLS AND THEIR SURROUNDING STROMA

*Interactions between soluble factors and their receptors dictate gene expression profiles in cancer cells and their surrounding stroma.* The mediation of stromal-epithelial interactions in the normal and malignant prostatic environment involves a number of soluble factors and their receptors. Soluble factors can serve paracrine, autocrine or intracrine functions with their actions mediated by their respective receptors or interactive partners. Soluble factors could mediate in a reciprocal manner the intercellular communication between stroma and epithelium that controls normal prostate development, benign enlargement of the prostate gland and its neoplastic transformation. The constellation of secreted soluble factors by different tissues may serve as chemoattractants or local growth inducers via appropriate cancer cell surface receptors for the secondary sites of cancer metastasis. Soluble factor communication is often bidirectional between stroma and epithelium, and it is coordinated with other signaling molecules, such as extracellular matrices and integrins, and other intracellular receptor signaling (eg steroid receptor). In some cases additive or synergistic interactions could occur between various signaling cascades that could culminate to create a vicious cycle of positive feedback, facilitating aggressive local cancer growth and metastatic spread to distant sites. Table 2 lists the most commonly cited pathways involving soluble factors. New insights into several of these pathways have provided exciting, potential therapeutic targets.

Recruitment of neovascular endothelial cells to proliferating cancer cells is thought to be required for the maintenance and stimulation of tumor growth. Thus, it is not surprising that the expression of vascular endothelial growth factor (VEGF) and receptors is tightly regulated by androgen. The secretion of

VEGF has been shown in glandular and surrounding stromal cells. The resulting effect is the stimulation of vasculogenesis by the action of VEGF on the endothelial component of the mesenchyma. Androgen deprivation has been shown to decrease VEGF expression by prostate cancer and it is thought to be a mechanism of castration-mediated apoptosis. Additionally, finasteride, a 5  $\alpha$ -reductase inhibitor, has been shown to associate with a decrease VEGF expression as well as microvessel density in clinical benign prostatic hyperplasia specimens. Direct inhibition of VEGF mediated angiogenesis by thalidomide is another mechanism affecting this pathway that is currently under clinical investigation.

Dysregulation of the interleukin-6 (IL-6) pathway has been found in autoimmune disorders as well as in different types of malignancy, including multiple myeloma and prostate cancer. The complexities of the IL-6 signaling pathway were detailed in a recent review. High levels of IL-6 secretion from prostate fibroblasts and smooth muscle cells as well as tumor cells themselves are thought to be a mechanism of ligand independent activation of AR in prostate cancer cells. IL-6 mediated regulation of AR activation has been shown to occur via the PI3K-Akt, STAT3 and MAPK pathways, and it is proposed to be responsible for the androgen independent progression of human prostate cancer. Interference with IL-6 signaling is a potential means of modulating the growth of advanced prostate cancer. Studies using an anti-IL-6 monoclonal antibody have shown tumoricidal effects in a murine model.

The insulin-like growth factor-I (IGF-I) pathway has been shown to be involved with malignant transformation in various tissue types. Malignant prostate epithelial cells are sensitive to surrounding IGF-I levels regardless of their androgen sensitivity status. IGF-I over expression has been shown to drive neoplastic transformation of murine prostate epithelium, while antisense RNA to IGF-I receptor inhibits prostate cancer proliferation and invasion. Other manipulations of the IGF axis with therapeutic potential include increasing IGF binding protein expres-



TABLE 2. Most commonly cited soluble factor signaling pathways regulating prostate growth and differentiation

References	Soluble Growth Factor				Function	Regulation at Androgen Independent Progression
	Name	Source	Receptor	Receptor Location		
van Moorselaar et al: Mol Cell Endo, 197: 239, 2002	VEGF	Epithelium, stroma	VEGFR-1, 2	Epithelium, stroma	Angiogenic factor	Disease prognosis neg correlation
van Moorselaar et al: Mol Cell Endo, 197: 239, 2002	bFGF (FGF-2)	Stroma	FGF-2R	Epithelium, stroma	Angiogenic factor	Disease prognosis pos correlation
Lail-Treker et al: J Soc Gyn Invest, 5: 114, 1998 + Kundsen and Edlund: Adv Cancer Res, 91: 31, 2004	HGF/SF	Stroma	c-met	Epithelium	Stimulates cell growth	Disease progression pos correlation
Blanchere et al: J Steroid Biochem, 82: 297, 2002	TGF- $\beta$	Epithelium	TGF- $\beta$ I-III receptors	Stroma	Induces apoptosis, increases angiogenesis, stimulates stromal but inhibits epithelial cell growth	Augmented expression at androgen withdrawal
Djavan et al: World J Urol, 19: 225, 2001 + Prostate, 58: 41, 2004	IGF-I	Stroma	IGF-IR	Epithelium, stroma	Stimulates cell growth, blocks apoptosis	Up-regulation at disease progression
Royuela et al: J Pathol, 202: 41, 2004	IL-6	Epithelium, stroma	IL-6R, sIL-6R	Epithelium, stroma	Promote differentiation + apoptosis inhibition	Increasing IL-6 signaling during disease progression
Planz et al: J Urol, 166: 678, 2001	Keratinocyte growth factor (KGF) (FGF-7)	Stroma	Gp130 KGF-R	Epithelium	Stimulates cell growth	Stromal KGF expression responded to androgen

sion, which has been shown to induce apoptosis in prostate cancer cells.

Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, c-met proto-oncogene, were shown to be prevalently expressed by localized and metastatic prostate cancer. Experimental evidence suggests that HGF/SF and c-met downstream signaling may regulate prostate cancer growth and metastasis through enhanced IL-6, androgen receptor, extracellular matrix and integrin interaction. Platelet derived growth factor (PDGF) and its receptor PDGFR or c-Kit are expressed in human prostate cancer and vascular endothelial cells and their interaction with host osteoblasts could also have a role in regulating prostate cancer growth and their colonization in bone. This opens up the opportunity of evaluating imatinib mesylate (STI571 or Gleevec [Novartis Pharmaceuticals, East Hanover, New Jersey]), a tyrosine kinase inhibitor, which specifically blocks the cell surface PDGF receptor, PDGFR or c-Kit and its downstream signaling cascade. ErbB family members, including epidermal growth factor (EGF) receptor (EGFR), erbB2/neu HER2, erbB3 and erbB4, are known to have a role in prostate cancer progression through their interactions with a broad spectrum of soluble factors and their downstream converging signaling pathways. A large family of heparin bound growth factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), HGF/SF and heparin-binding EGF-like factor could be targeted either individually or collectively through the inactivation of their co-receptor, perlecan, a heparan sulfate proteoglycan. Farach-Carson et al provided experimental evidence by blocking perlecan with sequence specific ribozyme, greatly decreasing the ability of prostate cancer to grow in the skeleton (personal communication). The transforming growth factor (TGF)- $\beta$  family and its receptors have a pleiotropic role in prostate cancer. Since this growth factor family also affects EMT, angiogenesis, extracellular matrix turnover and host immune surveillance,

a large body of literature describes the possibility of targeting TGF- $\beta$  receptor pathways for altering prostate cancer behaviors.

Further understanding of the complexities of soluble factor mediated stromal-epithelial communication may yield novel therapeutic targets for prostate cancer. Manipulating these pathways in the native environment may prevent the development or growth of primary malignancies, while alterations in remote tissues may convert a previously fertile metastatic site into a hostile environment for tumor cells.

*Solid ECMs and integrin interactions promote cancer cell proliferation, survival and the ability to adhere, migrate and metastasize.* At the invasion front of tumor cell clusters a noticeable derangement of ECM barriers often occurs.<sup>19,30,31</sup> Interestingly most key enzymes controlling ECM breakdown are not derived from tumor cells, but rather from the host stroma, such as immune, inflammatory, endothelial and fibromuscular stromal cells. It is still unclear whether cancer cell invasion develops before or after interacting with host stroma, nor is it clear whether stroma response is subject to reciprocal regulation by cancer cells. Since the maintenance of epithelial homeostasis requires the participation of stroma, it is likely that when epithelium changes, the stroma inevitably follows.<sup>32</sup> Cancer cells are likely to evolve continuously under the influence of products from deranged ECM barriers. Interactions between the soluble factors, and/or degraded and released solid tissue matrix proteins and cancer cells become possible due to the breakdown of tumor stroma barriers. ECM and its degradative products could signal cancer cells through their cell surface integrin or nonintegrin associated receptors and affect cell behaviors, such as cell polarity, secretion, adhesion, motility and invasion, and integrated cell functions, such as proliferation, differentiation and survival. Integrins may have more complex roles by coordinating their actions with metalloproteinases and serine proteases, which together may increase cancer cell



invasion, migration and extravasation into secondary sites of cancer growth.<sup>31,32</sup> The preferential use of certain integrin isotypes,<sup>33</sup> the cross-talk between soluble and solid matrix factors, cell contact in a 3-D structural scaffold and downstream signaling pathways in prostate cancer cells during androgen independent and bone metastatic progression could potentially reveal new therapeutic targets for cancer metastasis therapy. New therapeutic agents in the form of an antibody or peptide have been developed based on their ability to interfere with ECM and cell-surface integrin interactions. Others were developed based on their interference with downstream converging signaling pathways originating from the soluble and solid tissue matrix mediated signaling that determines cancer cell proliferation, survival and sensitivity toward drugs, hormones and/or radiation.

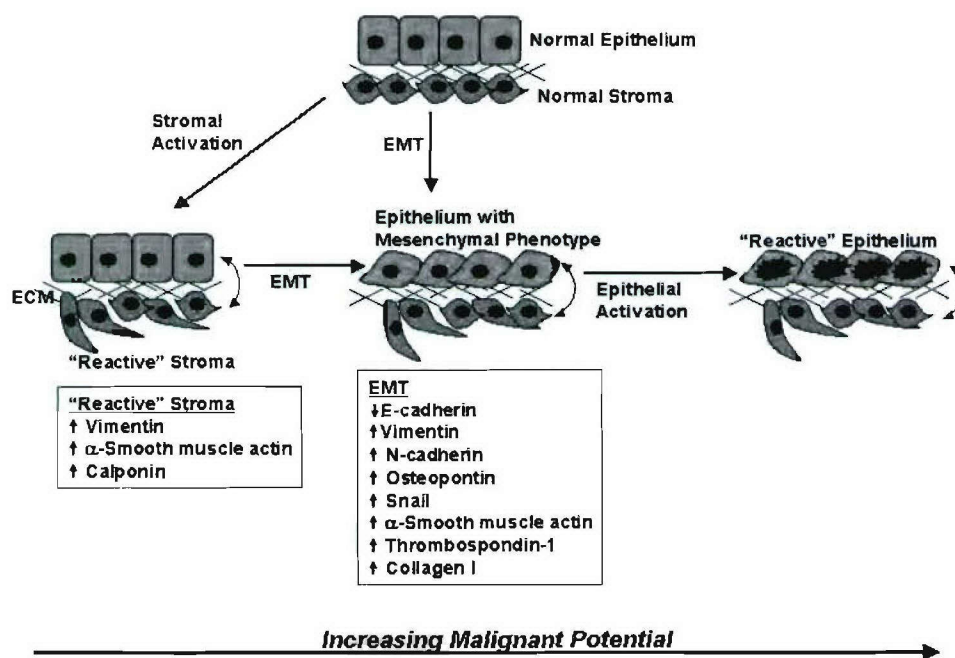
#### TRANSDIFFERENTIATION OF PROSTATE CANCER CELLS TO INCREASED MALIGNANT POTENTIAL BY EMT

EMT is a fundamental process that determines body plane and polarity during embryonic development, where epithelial cells in the ectoderm migrate, undergo mesenchymal transition, invade and insert themselves between ectoderm and endoderm layers.<sup>16</sup> EMT has been documented in breast, bladder and prostate cancers through increased expression of mesenchymal genes and it is associated with increased cell motility, invasion and migration.<sup>34–36</sup> We and others have observed increased expression of vimentin and N-cadherin but decreased expression of E-cadherin during prostate cancer progression to invasive phenotypes through *HER-2/erbB2/neu* oncogenic transformation or through in vivo clonal interactions.<sup>37–39</sup> A number of soluble factors, such as EGF/TGF $\alpha$ , TGF $\beta$ 1 and acidic FGF, and solid matrix, such as collagen, have been shown to induce EMT and associated phenotypic changes.<sup>16,35,40</sup> The resulting cancer cells with mesenchymal

phenotypes can secrete matrix metalloproteinases, become more responsive to the inductive growth factor and cytokine milieu in the surrounding tumor microenvironment and acquire increased malignant potential by augmenting cell motility, migration and invasion. The figure shows stromal activation, EMT and its downstream epithelial activation in response to inductive cues from the cancer microenvironment, possibly mediated by activation of mitogen activated protein kinase/extracellular signal-regulated kinase, Src and PI3K activity, thus, affecting cancer cell proliferation, survival, motility and invasion.<sup>16,41</sup> Targeting EMT downstream pathways has been shown to reverse the morphological and biochemical features of EMT in cancer cells, restore their differentiation and decrease cancer metastasis.<sup>42,43</sup>

#### REACTIVATION OF STROMAL FIBROBLASTS TO MYOFIBROBLASTS DURING CANCER PROGRESSION

Another morphological and biochemical transition in cancer associated stromal compartments, called reactive stroma, develops during disease progression (see figure). This process is shared by wound healing and tumorigenesis,<sup>32,44</sup> in which stromal fibroblasts are observed to undergo myofibroblastic transition morphologically and biochemically. Although the origin of myofibroblasts remains controversial, it is heterogeneous and they may be recruited from host fibroblasts, vascular smooth muscle cells and pericytes.<sup>32</sup> Tuxhorn et al provided evidence that reactive stroma respond to prostate cancer epithelium, resulting in increased expression of vimentin, smooth muscle  $\alpha$ -actin and calponin, characteristic of the myofibroblast phenotype, in the surrounding cancer associated stroma.<sup>45</sup> De Wever and Mareel comprehensively reviewed the interplay between cancer and stroma, and proposed that 2 tightly interactive pathways, called the efferent



Transition of normal prostate epithelial cells to increased malignant potential through cellular interaction with host stromal cells. There are potentially 3 pathways by which normal prostate epithelial cell can progress to become cancer cell with uncontrolled growth and probability to disseminate. 1) Stromal activation to become reactive stroma could be responsible for driving premalignant prostate epithelial cell to undergo additional phenotypic and genotypic changes, so that it acquires proliferative and survival advantages in primary organ. 2) Under instruction from secreted soluble and insoluble matrix factors in microenvironment premalignant epithelial cell can undergo EMT by expressing mesenchymal genes and gaining increased invasion, migration and metastatic potential. Epithelial cell often appears in cancer invasion front, become amoeba-like, loses cell polarity and gains invasive and migratory properties in response to chemical and haptotactic stimuli from immediate cancer microenvironment. 3) Additional genotypic and phenotypic changes may occur in prostate epithelial cells through epithelial activation process. In response to continued inductive cancer microenvironment milieu at primary or secondary growth site reactive epithelium could be lethal clone of prostate cancer cells. Understanding cancer and microenvironment interaction at cellular and molecular levels could greatly help us in designing rational therapies for treatment of human prostate cancer metastasis.



and afferent pathways, ultimately determine the nature of the reciprocity between cancer and stroma.<sup>32</sup>

In the efferent pathway cancer cells trigger a reactive stromal response. Cancer cells could induce stromal response by the release or deposit of soluble factors, such as TGF $\beta$  and PDGF, in the cancer associated microenvironment. In response to these factors stromal fibroblasts undergo myofibroblast transition. This pathway appears necessary and it could represent an early event in prostate and breast cancer progression. Evidence in support of this suggestion comes from the observation that normal stromal fibroblasts from fetal urogenital sinus (ie embryonic prostate fibroblasts<sup>46</sup>) and mammary gland (isolated from reduction mammoplasty<sup>47</sup>), when co-inoculated with their respective prostate or breast tumor cells in vivo, inhibited their growth. In contrast, spontaneously immortalized prostate stromal cells<sup>20</sup> or cancer associated (but not benign associated) stromal cells<sup>48</sup> co-inoculated with prostate cancer cells in vivo induced their growth, suggesting possible roles of stromal cells after activation or transdifferentiation. In these cases normal stromal cells inhibited cancer growth through the induction of cancer cell differentiation, whereas transdifferentiated or altered stromal cells (reactive stroma) promoted tumor growth and accelerated androgen independent and bone metastatic progression. The growth stimulatory mode of stromal cells could result from the conversion or transdifferentiation of stromal fibroblasts to a reactive stromal population via the proposed efferent pathway.

Somewhat unclear in these models is whether the stromal reaction in response to tumor epithelial induction involves permanent genotypic changes. Intriguing experimental evidence indicates that permanent genetic changes occurred in tumor associated stroma harvested from breast cancer specimens by laser captured microdissection.<sup>49</sup> Mouse stromal cells outgrown from human prostate xenografts were reported to have consistent and identical chromosomal aberrations.<sup>50</sup> Taken together these results strongly support the possibility that interaction with cancer cells can cause permanent genetic changes in cancer associated stromal cells. This suggestion is supported experimentally by our co-culture study, in which growing C4-2, an androgen independent but not parental androgen dependent human prostate cancer LNCaP cell line together with the human MG-63 osteosarcoma cell line under 3-D conditions produced consistent and stable chromosomal changes and phenotypic gene expression, as described.<sup>27</sup>

The afferent pathway describes the cancer cell response to altered stromal cells in the cancer microenvironment. Several key effects of myofibroblasts or reactive stroma on cancer cell behaviors have been suggested. Reactive stromal cells can exert a pro-invasive signal, increasing the motility and invasion, and decreasing the apoptosis of cancer cells. They can mediate cancer pain through the release of cytokines or neuroendocrine factors. They can guide cancer cell perineural invasion and dissemination through the release of soluble and solid matrix factors.<sup>32</sup> All of these cancer phenotypes have been observed in human prostate cancer, underscoring the importance of this pathway in human prostate cancer progression. Exploring the intimate interactions between tumor and stroma, and the dynamic transition of stromal cells during cancer progression could lead to more accurate targeting of this molecular process and new treatment protocols for prostate cancer metastasis.

#### THE SWITCH OF OSTEOMIMICRY AND VASCULOGENIC MIMICRY UPON METASTATIC PROGRESSION

Many features of cancer growth, development and dissemination are known to recapitulate embryogenesis. This is not surprising since cancer cells are pluripotent and have stem cell-like properties capable of differentiating into and ex-

pressing phenotypes restricted to specialized cell types, such as bone or endothelial cells. During cancer progression prostate or breast cancer cells can switch their gene expression profiles by mimicking bone<sup>10</sup> or endothelial<sup>12</sup> cells. This section reviews the biology and potential implications of osteomimicry and vasculogenic mimicry in cancer growth and metastasis.

Several proteins commonly associated with and/or restricted to bone cells, such as osteocalcin (OC), bone sialoprotein, osteopontin, osteonectin (ON or SPARC), osteoprotegerin (OPG), parathyroid hormone-like related protein, macrophage colony-stimulating factor, receptor activator of nuclear factor  $\kappa$ B and receptor activator of nuclear factor  $\kappa$ B ligand, were also found to be expressed by prostate cancer cells.<sup>11,51-56</sup> Moreover, a bone homing metastatic human prostate cancer cell line of LNCaP lineage, C4-2B, was found to calcify in vitro under mineralizing conditions.<sup>56</sup> The acquisition of new gene expression profiles by prostate cancer cells that mimic bone is called osteomimicry.<sup>10</sup> Apparently this interaction is reciprocal. The gene expression profiles of normal cells surrounding the cancer epithelium at the primary (eg reactive stroma) and the metastatic bone sites were also found to undergo morphological and biochemical changes.<sup>26,57</sup> Using the human osteosarcoma cell line MG-63 as a model we observed permanent genetic, morphological and gene expression changes in MG-63 cells after exposure in vitro to an LNCaP lineage, androgen independent human prostate cancer cell line, C4-2, in co-culture under 3-D conditions.<sup>26,57</sup> These observations show the plasticity of cancer cells and their surrounding stroma, and the potential reciprocal inductive influences between them. More information is needed to determine the true inductive potential and plasticity of normal epithelium and stroma when exposed to cancer cells and the possible effect of aging and contribution by circulating stem cells to this cellular interaction process since prostate cancer is known to develop more frequently in the aging population and circulating stem cells have been found to reside in and have key roles affecting the growth and differentiation of many adult organs, including bone and liver. Further understanding of epithelial-stromal interaction in the context of cancer development and progression would improve our capability to design therapies targeting the tumor and tumor microenvironment interphase.

To understand the molecular basis of osteomimicry groups at our laboratory focused on investigating the mechanism of osteocalcin promoter switching during human prostate cancer progression. These studies revealed that activation of transcription factors, Runx2, JunD/Fra-2 and Sp1, could have a role.<sup>51</sup> It has been shown that a number of intracellular signaling pathways, including cyclic adenosine monophosphate responsive G protein coupling and vitamin D receptor mediated pathways, could be involved in the activation of OC promoter activity.<sup>51,58,59</sup> By exposing target cells to soluble growth factors, such as bFGF, parathyroid hormone and the rich bone matrix protein collagen 1, OC promoter activation was observed. Since these soluble and matrix protein factors are deposited by cancer and their associated prostate or bone stromal cells in the microenvironment, we suggest that the activation of intracellular signaling pathways and a coordinated switch of transcription factors may occur during prostate cancer progression and serve as a likely molecular basis of osteomimicry in prostate cancer cells. The expression of bone-like proteins could confer certain advantages to prostate cancer cells. 1) Prostate cancer cells could behave like osteoblasts by participating directly in osteoclastogenesis, where increased bone turnover is observed.<sup>10</sup> This increased local bone turnover following prostate cancer bone colonization creates new sites to facilitate the further growth and metastasis of prostate cancer cells. 2) Expression of OC and bone sialoprotein by prostate cancer cells could enhance bone mineralization. These bone



matrix proteins are capable of binding with high affinity to the mineral component of the bone, hydroxyapatite, and participate in the recruitment of osteoclasts and osteoblasts, which affect bone resorption and deposition, respectively. 3) Osteomimicry may be required for prostate and breast cancer growth and survival in bone. Studies of prostate, breast and several other cancer types<sup>60</sup> indicated the ability of bone matrix proteins to confer increased cancer cell growth, adhesion, migration and invasion.<sup>64,61,62</sup> The engagement of integrins  $\alpha\beta3$  and  $\alpha\beta5$  by the bone matrix protein SPARC resulted in enhanced VEGF production by prostate cancer cells.<sup>63</sup> De et al proposed that VEGF and VEGF receptor-2 interaction through an autocrine loop stimulated prostate cancer cell growth and integrin activation further to increase prostate cancer cell adhesion and migration toward bone.<sup>63</sup> Experimental tumor models inoculating an osteoblastic human prostate cancer cell line, C4-2B,<sup>55</sup> or an osteoblastic human breast cancer cell line, ZR-75-1,<sup>64</sup> in immune compromised mice revealed that targeting the interphase between cancer cells and bone using osteoprotegerin or an endothelin A receptor antagonist, Astrasentan (Abbott Laboratories, Abbott Park, Illinois), resulted in selective tumor shrinkage and decreased osteoblastic reaction in bone, respectively, but did not affect tumor growth at other ectopic and orthotopic sites. These results suggest that cross-talk between cancer and bone specifically used the osteomimetic properties of prostate and breast cancer cells, and such communication is different from cancer interaction with its primary organ sites (eg prostate and breast stromal environments). If so, then it is not surprising that a bone directed targeting strategy would be highly selective and effective against only bone metastasis and such a strategy would be ineffective against the growth of prostate cancer at the primary organ site or metastases to other visceral organs, as described. To improve further the therapeutic targeting of prostate cancer local growth and distant metastasis a more comprehensive definition of organ specific stromal microenvironments in the primary and visceral organs is urgently needed.

Vasculogenic mimicry describes the plasticity by which melanoma cells, when placed under ischemic conditions, form their own blood supply channels connecting with previously existing vasculature, express endothelial associated markers (vascular endothelial cadherin, CD34 and endothelial cell specific molecule) and form an ECM rich vasculogenic network in 3-D culture.<sup>12</sup> These properties were first described by Hendrix et al using a melanoma model.<sup>12</sup> It appears that vasculogenic mimicry occurs in several other cancer types, including prostate cancer. The molecular mechanisms underlying vasculogenic mimicry by melanoma cells were investigated using microarray analyses and inhibitor studies. The results of these studies showed that tyrosine kinase EPHA2, vascular endothelial cadherin, focal adhesion kinase and PI3K are involved as integrated signaling pathways controlling vasculogenic mimicry.<sup>12</sup> Since ischemia facilitates vasculogenic mimicry, it is likely that hypoxia, a condition commonly associated with tumor progression, may promote this morphological and functional transition of cancer epithelial cells.

Anti-angiogenic therapy for cancer has not been highly effective despite the well appreciated fact that tumor angiogenesis is a prerequisite for tumor growth. The origin of the endothelial network formed around tumor cells and its relative sensitivity to anti-angiogenic therapy is an important therapeutic concern. Realizing that one of the potential contributing cell types for tumor angiogenesis is the tumor itself plus other cells not of obvious endothelial lineage may provide a clue to the development of anti-angiogenic drugs targeting the heterogeneous origin of the endothelial network in the tumor microenvironment. Vasculogenic mimicry in cancer must be further explored, so that more effective anti-angiogenic therapies can be developed.

#### THREE-D TISSUE ORGANIZATION REGULATES THE POLARITY, SECRETION, GENE EXPRESSION AND BEHAVIOR OF CANCER CELLS

Cancer in situ does not grow as a 2-D adherent cell array, but rather as a 3-D, closely packed organoid in close interaction with its microenvironment. A number of 3-D experimental models have been developed to assess how cell microenvironment regulates cell polarity, genetics and behavior.<sup>17,19,65-67</sup> Malignant breast cancer cells cultured on plastic yield the same phenotype and growth rate as nonmalignant breast epithelium. However, when placed in a 3-D reconstituted extracellular matrix scaffold, malignant breast epithelial cells form an amorphous structure with unregulated proliferation, due in part to the aberrant expression of integrins and EGFR, and loss of the ability to sense contextual cues from the surrounding microenvironment. In comparison, nonmalignant breast epithelial cells undergo growth arrest, form a polarized alveolar structure and secrete milk.<sup>65,68</sup>

A 3-D assembly of cells can be conveniently constructed by growing prostate cancer and prostate or bone stromal cells under zero gravity simulated conditions.<sup>27,67</sup> Using this model system, our laboratory have shown the reciprocal interaction between prostate cancer and bone stromal cells, whereby permanent and stable phenotypic and genotypic changes occur in prostate epithelial and bone stromal cells after cellular interaction under zero gravity simulated conditions.<sup>27</sup> For example, the nontumorigenic human prostate cancer cell line, LNCaP, can be promoted to undergo androgen independent progression and express a highly malignant potential, including the ability to invade locally and metastasize to the skeleton, only after cellular interaction with prostate or bone stromal cells, or an extracellular matrix scaffold under 3-D growth conditions. This is remarkable considering that none of these changes were observed when co-culturing these cells under 2-D conditions. These findings recapitulate our previous observations in vivo, when we observed permanent phenotypic and genotypic changes in prostate cancer epithelial cells, including androgen independence and bone metastasis, when co-inoculated and grown together with bone stromal cells as 3-D chimeric tumors.<sup>25,69</sup>

In addition to the breast and prostate cancer models described, developing kidney, cartilage, heart, pancreas and ovary under 3-D conditions recapitulates the normal patterns of differentiation of these organs, suggesting again the parallelism between organ development and carcinogenesis. The 2 models require committed cellular interaction under 3-D conditions. Although the precise mechanism of cellular interaction between cancer cells and soluble factor(s)/ECMs in the microenvironment remains speculative, most likely the underlying molecular basis controlling the progression of cancer resides in certain coordinated changes of gene transcription that occur in a highly temporal and spatially regulated manner in response to inductive cues from the host microenvironment.<sup>19,68</sup> In a recent review Ingber summarized the principle of mechanochemical transduction of signals to control normal and malignant tissue differentiation, in which mechanical stretch of cells can trigger a host of coordinated chemical signals that could control cell growth, the expansion of basement membranes, and the orderly progression of morphogenesis and cytodifferentiation of normal developing tissues.<sup>19</sup> In carcinogenesis deregulated epithelial-mesenchymal interaction could cause accelerated turnover of basement membrane proteins and the release of mechanical constraints on cancer epithelium, so that a deregulated growth, migration and differentiation program can ensue.

#### PROSTATE CANCER SKELETAL METASTASIS: TARGETING TUMOR AS WELL AS ITS MICROENVIRONMENT

Men with advanced prostate cancer experience debilitating bone metastatic disease for which there is no curative therapy. To develop a sound approach tackling prostate cancer bone metastasis many laboratories have begun to evaluate



the biology and molecular basis of prostate cancer bone metastasis. Using human prostate cancer cell lines and xenograft models we can conclude that 1) bone metastasis is conferred by specific cellular interaction between cancer and host bone cells, in which the growth and differentiation of prostate cancer and bone stromal cells can be reciprocally regulated,<sup>26,57,70</sup> and certain chemoattractants produced by bone cells to recruit and retain prostate cancer cells in bone,<sup>71</sup> 2) metastatic prostate cancer cells can express bone-like proteins (osteomimicry), participate in osteoclastogenesis and proliferate, invade and survive in the bone microenvironment,<sup>10</sup> 3) prostate cancer progression to androgen independence and bone metastasis is accelerated by androgen withdrawal and 4) targeting bone and the interphase between prostate cancer and bone has yielded improved survival in mice with prostate cancer xenografts<sup>11</sup> and patients with prostate cancer.<sup>72</sup> Table 3 lists clinical experience with various therapeutic strategies co-targeting cancer and its microenvironment with references.

EGFR emerged as an initial critical target. In an effort to repeat the success seen in other tumor types with gefitinib, a small molecule that inhibits the kinase activity of EGFR, a phase II trial assessed the efficacy of single agent gefitinib in patients with advanced hormone refractory prostate cancer (HRPC) but failed to show a significant response rate. The success of trial design was affected by patient selection and it could be improved by obtaining additional data on the status of EGFR mutations since it has been reported that tumors containing mutated EGFR are more sensitive to growth inhibition by gefitinib.

PDGFR has emerged as a dual target for epithelial cancer cells and bone stroma. PDGF and PDGFR are co-expressed in prostate cancer and the neovasculature at metastatic sites. The binding of the ligand (PDGF) to its receptor (PDGFR) results in apoptosis inhibition but it also accounts for the osteotropism of prostate cancer.<sup>10</sup> Imatinib mesylate (Gleevec) is a small molecule that binds to an adenosine triphosphate binding site of PDGFR kinase and blocks this particular signaling pathway. A phase I trial of imatinib plus docetaxel showed no single agent activity of imatinib but potential synergy for the combination. This concept is currently being addressed in a phase II randomized placebo controlled trial. Of note, preclinical data suggest that PDGFR- $\beta$  kinase is a major mediator for increased tumor interstitial pressure, which hinders the adequate delivery of cytotoxics to the targeted epithelial component. Imatinib given with paclitaxel effectively decreased tumor in-

terstitial pressure, which in turn led to increased uptake of paclitaxel and enhanced tumor cell kill.

Bisphosphonates are a class of drugs that target osteoclasts and have been used with great success in disease states associated with increased osteolytic activity (osteoporosis and multiple myeloma). It is now appreciated that increased osteolysis is present in metastatic prostate cancer, and so the osteoclast may be a prime target. Preclinical data with the third generation bisphosphonate zoledronic acid (Zometa, Novartis Pharmaceuticals) appear promising in that regard. Currently clinical interest in bisphosphonates has focused primarily on the prevention/delay of skeletal related events (fractures, need for therapeutic intervention and cord compression) and control of bone pain. A phase III trial of zoledronic acid in patients with metastatic hormone refractory prostate cancer showed that the drug decreased the absolute risk of skeletal related events by 11% at a median followup of 24 months. This led to Food and Drug Administration approval for that particular indication. Of note, pamidronate has failed to provide equivalent results.

Atrasentan is a small molecule that blocks the receptor that mediates the effects of endothelin-1. Endothelin-1 is a potent mitogen for osteoblasts and it modulates nociception, thus, providing a target for the cancer-osteoblast interphase and control of bone pain. A prospective randomized, placebo controlled, phase II trial investigated the efficacy of atrasentan at 2 doses (2.5 and 10 mg orally daily) for delaying time to progression (TTP) and providing adequate analgesia in patients with metastatic HRPC. Atrasentan at 10 mg vs placebo was found to prolong median TTP (183 vs 137,  $p = 0.13$ ) with a similar result for the 2.5 mg dose. Analysis of the data by actual treatment received (244 of 288 patients) showed a statistically significant difference in median TTP (196 vs 129 days,  $p = 0.021$ ) in favor of atrasentan. Median time to prostate specific antigen progression was twice as long in the 10 mg atrasentan group than in the placebo group (155 vs 71 days,  $p = 0.002$ ). Quality of life measurements were not significantly improved in the treated arms. A phase III trial of atrasentan has completed data accrual and results are awaited.

The idea of bone and tumor co-targeted therapy has been tested. A gene therapy trial focused on the recombinant, replication defective adenovirus Ad-OC-TK (OC promoter-driven-herpes simplex virus-thymidine kinase co-expressed in both tumor and stromal cells) resulted in effective tumor lysis in preclinical models and in patients in a phase I trial. A phase II trial was done in patients with hormone refractory

TABLE 3. Clinical experience with targeting cancer and its microenvironment

Drug	Target	Trial Phase	Comment	References
<b>Soluble factors:</b>				
Gefitinib	EGFR	II	Min single agent activity	Moore et al: Ann Oncol, 5: 326, 2002
Trastuzumab	Her-2-neu	II	Trial ongoing, target of questionable clinical significance	Uehara et al: J Natl Cancer Inst, 95: 458, 2003
Imatinib mesylate	PDGFR- $\beta$	I	No activity as single agent, possible synergy with taxanes	Mathew et al: J Clin Oncol, 22: 3323, 2004
<b>Bone targeted:</b>				
Zoledronic acid	Osteoclast	III	SRE 11% absolute risk decrease, Food + Drug Administration approved for HRPC	Saad et al: J Natl Cancer Inst, 94: 1458, 2002
Atrasentan	Osteoblast, PC	II	Trend toward delayed TTP	Nelson et al: Natl Med, 1: 944, 1995 + Carducci et al: J Clin Oncol, 21: 679, 2003
Strontium <sup>89</sup>	Bone interface	II	Prolongs overall survival in responsive HRPC when used as consolidation	Tu et al: Lancet, 357: 336, 2001
Gene therapy	Osteoblast, PC	I	Concurrent co-targeting to tumor + stroma using osteocalcin promoter	Kubo et al: Human Gene Ther, 14: 227, 2003
<b>Angiogenesis:</b>				
Bevacizumab	VEGF	II	Min single agent activity	Reese et al: Proc ASCO, 1999
Thalidomide	bFGF, IL-8	II	Min single agent activity, possible synergy with taxanes	Figg et al: Clin Cancer Res, 7: 1888, 2001 + Semin Oncol, 28: 62, 2001
GVAX immunotherapy	Unknown	II	Antitumor effects, decreased osteolytic activity	Simons et al: Proc ASCO, 2003



prostate cancer who received KAVE chemotherapy (ketoconazole/doxorubicin alternating with vinblastine/estramustine) and were then randomized (those who responded or were clinically stable) to continue with doxorubicin alone or doxorubicin plus strontium<sup>89</sup>. The arm that received the chemotherapy/radiopharmaceutical combination had a median survival of 27.7 vs 16.8 months in those who received doxorubicin alone ( $p = 0.0014$ ). These findings await confirmation from a phase III trial that is currently underway.

Angiogenesis has been proposed as a stromal target that can limit further progression of metastatic prostate cancer. A phase II trial of bevacizumab, a monoclonal antibody that targets VEGF, failed to produce significant clinical activity when used as single agent in patients with HRPc. Thalidomide has also shown anti-angiogenic effects along with immunomodulatory activity. A phase II trial of single agent thalidomide indicated minor activity when used alone. Possible synergy with chemotherapy has also been proposed.

Finally, active immunotherapy may also target the stroma. A phase II trial of GVAX (Cell Genesys, South San Francisco, California), an allogeneic vaccine ex vivo transduced to secrete granulocyte-macrophage colony-stimulating factor, was completed. It was noted that carboxy-terminal telopeptide of type I collagen, a marker of osteolytic activity (typically increased in patients with HRPc) remained stable or decreased in 70% of GVAX treated patients, thus, raising the issue of additional, as yet unidentified targets in the bone stroma.

#### CONCLUSIONS

A number of targets that facilitate the cross-talk between stroma and prostate cancer cells have emerged. Combining therapeutic strategies that co-target 2 or more of these targets with or without concomitant cytotoxic strategies will usher in a new era of drug development in metastatic prostate cancer. The goal, which is quite an attainable one, will be to delay the symptomatic progressive metastatic phenotype and convert HRPc into a chronic disease, in which host stromal cells and invading prostate cancer cells have learned to coexist in equilibrium.

#### FUTURE PROSPECTIVE

Cancer genotype and phenotype are influenced profoundly by the microenvironment. Permanent genetic and behavioral modifications have been observed in cancer and stromal cell compartments upon co-culture of these cells under 3-D conditions without the need of transferring foreign genes. Through reciprocal cellular interaction these stably induced rather than inherited genetic and phenotypic changes can contribute to cancer progression. If the underlying assumption that cancer-microenvironment interaction dictates cancer progression is correct, this provides a molecular basis and immeasurable opportunities for therapeutic intervention to change the natural history of prostate cancer. Future pursuit of cancer-stromal interaction and altered stromal signature at the molecular level during disease progression will help improve our ability to diagnose, prognose and treat cancer. Ultimately profiling the molecular signatures of cancer as well as its associated stromal components could provide new insights that will be applied and practiced in the future as the basis for personalized medicine.

Drs. Valerie Odero-Marah and Wen-Chin Huang provided artwork and discussions. Gary Mawyer and Melinda Russo assisted with the manuscript.

#### APPENDIX

Bhowmick et al recently reported that interrupting TGF $\beta$  signaling in prostate stroma modulates the oncogenic potential of adjacent epithelium.<sup>73</sup>

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# Cotargeting tumor and tumor endothelium effectively inhibits the growth of human prostate cancer in adenovirus-mediated antiangiogenesis and oncolysis combination therapy

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Tumor–endothelial interaction contributes to local prostate tumor growth and distant metastasis. In this communication, we designed a novel approach to target both cancer cells and their “crosstalk” with surrounding microvascular endothelium in an experimental hormone refractory human prostate cancer model. We evaluated the *in vitro* and *in vivo* synergistic and/or additive effects of a combination of conditional oncolytic adenovirus plus an adenoviral-mediated antiangiogenic therapy. In the *in vitro* study, we demonstrated that human umbilical vein endothelial cells (HUVEC) and human C4-2 androgen-independent (AI) prostate cancer cells, when infected with an antiangiogenic adenoviral (Ad)-Flk1-Fc vector secreting a soluble form of Flk1, showed dramatically inhibited proliferation, migration and tubular formation of HUVEC endothelial cells. C4-2 cells showed maximal growth inhibition when coinfecting with Ad-Flk1-Fc and Ad-hOC-E1, a conditional replication-competent Ad vector with viral replication driven by a human osteocalcin (hOC) promoter targeting both prostate cancer epithelial and stromal cells. Using a three-dimensional (3D) coculture model, we found that targeting C4-2 cells with Ad-hOC-E1 markedly decreased tubular formation in HUVEC, as visualized by confocal microscopy. In a subcutaneous C4-2 tumor xenograft model, tumor volume was decreased by 40–60% in animals treated with Ad-Flk1-Fc or Ad-hOC-E1 plus vitamin D<sub>3</sub> alone and by 90% in a combined treatment group, compared to untreated animals in an 8-week treatment period. Moreover, three of 10 (30%) pre-established tumors completely regressed when animals received combination therapy. Cotargeting tumor and tumor endothelium could be a promising gene therapy strategy for the treatment of both localized and metastatic human prostate cancer.

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Hormone-refractory prostate cancer is one of the leading causes of cancer mortality and morbidity in North American men.<sup>1</sup> Despite aggressive efforts toward earlier detection and treatment, the mortality resulting from distant metastasis of prostate cancer remains high. The most common site of prostate cancer metastasis is bone, with skeletal metastases identified at the time of autopsy up to 90% in patients dying from prostate cancer.<sup>2,3</sup> While localized prostate cancer may be cured, patients with advanced hormone refractory and bone metastatic cancer have a poor prognosis and a median survival time of 4 months.<sup>4,5</sup> Conventional therapies such as hormone therapy, radiation therapy and chemotherapy assume that cancer is a clonal cell disease and that

targeting tumor epithelium can best control tumor growth. These forms of therapy are highly effective but unfortunately not longlasting. Patients eventually develop hormone-refractory disease resistant to conventional therapeutic interventions. New therapeutic approaches to hormone-refractory prostate cancer are urgently needed.

The growth and metastasis of prostate cancer cells are intimately affected by their microenvironment. Well-established autocrine, paracrine and endocrine communication loops exist between cancer cells and their adjacent cellular components,<sup>6</sup> including (1) smooth muscle cells and fibroblasts, which provide critical soluble growth factors and extracellular matrices (ECMs) that support tumor growth, survival and differentiation; (2) endothelial cells, which form critical blood vessels, supply oxygen and nutrients to the tumor epithelium, and remove metabolic wastes from the tumor cells; (3) inflammatory cells, which could support important cytokines to defend against bacterial infection of the tumor epithelium and also potentially maintain the

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growth requirements of tumor cells. Using an experimental coculture cell model and chimeric tumor model<sup>7</sup> comprised of human prostate cancer and bone stromal cells to mimic a functional prostate organoid, our laboratory designed a strategy cotargeting both tumor and its supporting stroma. Recent studies with bone and directed targeting of hormone refractory prostate cancer using a combination of chemotherapy (which targets prostate tumor epithelium) plus strontium 89 (which targets bone), and in an experimental model of prostate cancer skeletal metastasis using a cotargeting strategy with conditional replication-competent adenoviral vector in which viral replication in both tumor and stromal cells was controlled by a tissue-specific and tumor restrictive promoter, osteocalcin (OC), has shown great promise, substantially improving patient survival<sup>5</sup> and curing animals with pre-existing prostate tumor in the skeleton.<sup>8</sup> These findings suggest that a cotargeting strategy could ultimately improve the treatment of hormone-refractory human prostate cancer and bone metastasis.

Extensive studies by many investigators have established the central role of tumor-associated angiogenesis in the invasion, growth and metastasis of solid tumors.<sup>9</sup> Tumor-associated angiogenesis can be effectively targeted as an anticancer therapeutic strategy.<sup>10–12</sup> Tumor cell behaviors can be greatly influenced by the balance of surrounding angiogenic stimulators and inhibitors<sup>13</sup> through paracrine-mediated regulation. Thus, antiangiogenic therapy can be used to try to stop new vessels from forming around a tumor and break up the existing network of abnormal capillaries feeding the cancerous mass.<sup>14</sup> One such approach could be to target the upregulated surface receptors on tumor endothelial cells.<sup>15</sup> Among these receptors, the vascular endothelial growth factor receptor 2 (VEGFR2, also known as Flk1) that binds the five isoforms of VEGF has a more restricted expression on endothelial cells and is upregulated in the tumor vasculature once these cells proliferate during neoangiogenesis.<sup>16</sup> Flk1 has been the subject of numerous studies in both animal models and in clinical trials.<sup>17–19</sup> Unfortunately, the clinical efficacy of single-agent antiangiogenic therapy has been disappointing.

To advance the cotargeting of both tumor and tumor microenvironment, we evaluated the responsiveness of prostate cancer to conditional replication-competent and VEGF receptor-based antiangiogenic therapy targeting three cell components: prostate tumor cells, prostate and bone stromal cells, and neovascular endothelial cells, in both cell culture and an experimental animal model. Our data demonstrated that adenovirus-mediated Flk1-Fc fusion protein delivery effectively suppress angiogenesis and tumor growth through a blockage of autocrine and/or paracrine mechanisms. Targeting prostate cancer cells with combined conditional oncolytic adenoviruses and antiangiogenic adenoviruses successfully achieved synergistic antitumor effects in human prostate cancer models, as described below.

## Materials and methods

### Cell lines and cultures

C4-2, an AI metastatic human prostate cancer cell line derived from LNCaP,<sup>20</sup> was grown in T medium (Invitrogen, CA) with 5% fetal bovine serum (FBS). HUVEC, a human umbilical vein endothelial cell line, was obtained from Cambrex Bio Science (Cambrex, CA) and maintained in endothelium-specific medium (EGM-2, Cambrex) according to the manufacturer's instructions. The cells were fed three times per week with fresh growth medium and maintained at 37°C in 5% CO<sub>2</sub>.

A three-dimensional (3D) coculture system was established to assess the effect of prostate cancer cells on endothelial tubular formation. A GFP-tagged C4-2 cell line (C4-2-GFP) was generated by infection of C4-2 with a recombinant retroviruses containing enhanced green fluorescence gene (EGFP) driven by a retroviral long terminal repeat (LTR) promoter. C4-2-GFP clones were obtained after selection of the transduced C4-2 cells by G418 (0.8 mg/ml). HUVEC cells were labeled with a red-fluorescent lipid dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), according to the manufacturer's instructions (Molecular Probes, OR). DiI-labeled HUVEC cells ( $5 \times 10^4$  cells/well) were plated on chamber slides, which were precoated with Matrigel (Becton-Dickinson, NJ). The Matrigel used in this experiment was diluted 1:1 in T-medium. Cells were allowed to settle for 1 hour and then the medium was carefully aspirated and replaced with 50  $\mu$ l of Matrigel. After polymerization (about 40 minutes), an overlay Matrigel containing  $5 \times 10^4$  C4-2/GFP cells preinfected with Ad-hOC-E1 or Ad-CMV-pA was added. Cell cultures were grown in EGM-2 plus T-medium with a ratio of 1:1 for 3 days.

### Adenoviral vectors

Ad-Flk1-Fc, an adenoviral (Ad) vector containing murine Flk1-Fc signal peptide followed by the ectodomain of murine Flk1 fused to the Fc fragment of murine IgG2a, and Ad-Fc carrying only the Fc fragment<sup>21</sup> were constructed by Dr Calvin Kuo at Stanford University (Stanford, CA). Replication-competent Ad-hOC-E1 and its replication-defective control Ad-CMV-pA were constructed by our laboratory as described previously.<sup>22</sup> All of the Ad vectors were amplified and purified according to the method of Graham and Prevec.<sup>23</sup> Viral titer was determined by plaque assay.

### Preparation of conditioned medium

$1 \times 10^6$  C4-2 and HUVEC cells were seeded in 60-mm culture dishes overnight and then subjected to infection with PBS (mock injection), or 10 MOI of Ad-Flk1-Fc or Ad-Fc. After 2 hours absorption, the viruses were removed and cells were cultured in fresh medium. After 48 hours culture, the conditioned media (CM) of HUVEC and C4-2 cells were harvested and concentrated by ultrafiltration with a Centricon YA10 (Millipore, MA). The amount of protein in CM was determined by BCA



Protein Assay kit (Pierce, IL) and then stored at  $-80^{\circ}\text{C}$  until use.

#### Western blot

A measure of  $20\text{ }\mu\text{g}$  of protein from CM or  $2\text{ }\mu\text{l}$  of mouse plasma was used for immunoblotting by the NOVEX (Invitrogen, CA) system. Membrane was probed with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-murine IgG2a Fc antibody (Southern Biotech, AL). Immunoreactive bands were revealed by the enhanced chemiluminescent (ECL) plus system (Amersham, NJ) according to the manufacturer's instructions and quantified using Quantity one-4.1.1 Del Doc gel documentation software (Bio-Rad, CA).

#### Cell proliferation and cytotoxicity assay

The HUVEC cell proliferation assay was performed using the MTT method. Briefly, 4000 HUVEC cells were seeded overnight on 96-well plates with EGM-2 medium containing  $10\text{ ng/ml}$  VEGF. Cells were subjected to infection with either Ad-Flk1-Fc or Ad-Fc at an MOI of 10, or incubation with  $10\text{ }\mu\text{g}$  of protein obtained from a concentrated CM of Ad-infected C4-2 cells for a period of 3 days followed by MTT assay. For the *in vitro* cytotoxicity assay,  $5 \times 10^5$  C4-2 cells were seeded overnight on a 24-well plate and then infected with 10 MOI of Ad-Flk1-Fc or Ad-Fc, and 2 MOI of Ad-hOC-E1 or Ad-CMV-pA alone or combination. After 2 hours absorption, the virus-containing medium was replaced with fresh growth medium for an additional 3 days of culture. The relative cell number was assessed by optical density (OD) at  $590\text{ nm}$  after crystal violet staining.

#### Tubular formation assay

Tubular formation on Matrigel was assayed as described previously<sup>24</sup> with a minor modification. Briefly, the 24-well plates were coated with  $200\text{ }\mu\text{l}$  Matrigel. HUVEC was trypsinized, washed and resuspended in EGM-2 medium in the presence or absence of CM ( $10\text{ }\mu\text{g/ml}$ ) from Ad-infected C4-2 cells. Cells ( $25,000\text{ cells}/100\text{ }\mu\text{l}$ ) were dispensed into each well and incubated for 8 hours. Each well was photographed under phase-contrast microscopy at  $\times 40$  magnification. The tubular formation was quantified by counting the number of connecting branches between discrete endothelial cells.

Tubular structures generated in 3D coculture were grown on chamber slides as described above and analyzed by LSM 510 META laser scanning confocal microscopy (Carl Zeiss, NY). Images of serial optical sections were taken at  $1\text{ }\mu\text{m}$  thickness in a basal-to-apical direction using a  $\times 10$  Neofluor objective.

#### Cell migration assay

HUVEC cell migration was performed using the wound healing method.<sup>25</sup> Briefly, a confluent monolayer of HUVEC cells grown on 24-well plates was wounded using a sterile  $200\text{-}\mu\text{l}$  plastic pipette tip. Displaced cells were removed with three washes, and fresh EGM-2

medium containing C4-2 CM was added. Photographs were taken under phase-contrast microscopy at 0, 12 and 24 hours after scraping. The position of the wound edge was noted against a reference grid and the migrating distance was measured using Openlab 3.0.8 software (Improvision, MA). Data were presented as the means of 10 measurements at each time point.

#### Animal studies

Male athymic mice (5 to 8-week old) (CD1 nu/nu) were purchased from Charles River (Wilmington, MA). The animals were kept under standard pathogen-free conditions and received care according to the criteria outlined in the National Academy of Sciences *Guide for the Care and Use of Laboratory Animals*. All animal experiments were approved by and complied with the regulations of Emory University School of Medicine.

Xenografts were established by subcutaneously injecting  $2 \times 10^6$  C4-2 cells in  $100\text{ }\mu\text{l}$  Matrigel into the flanks of mice. Tumor-bearing mice were designated as small, medium and large tumor groups based on their established tumor volume of  $\sim 50$ ,  $\sim 200$  and  $\sim 500\text{ mm}^3$ , respectively. In each group, the mice were randomized and given PBS (vehicle control),  $2 \times 10^9$  PFU of Ad-Flk1-Fc (intratumoral injection, twice per week for 2 weeks), or  $2 \times 10^9$  PFU of Ad-hOC-E1 (intravenous injection for single dose) alone or together. Mice receiving Ad-hOC-E1 were treated by intraperitoneal (i.p.) administration of  $100\text{ }\mu\text{l}$  vitamin  $\text{D}_3$  ( $4\text{ ng/dose}$ ) every other day for 3 weeks. Vitamin  $\text{D}_3$ -treated mice were fed a sterilized calcium-deficient diet (ICN Research Diets) as described previously.<sup>22</sup> Tumor volume measurements were taken weekly and calculated according to the formula:  $\text{length} \times \text{width}^2 \times 0.5236$ . Data are expressed as fold of the end point tumor volume in treatment groups relative to that in the vehicle control group. Blood samples were also obtained from Ad-Flk1-Fc-treated mice in the small tumor group every other week by saphenous vein puncture with heparinized capillary tubes after anesthesia. All animals were killed 8 weeks after treatment. Tumors were excised and then either embedded in OTC or fixed with 10% formalin for histomorphologic and immunohistochemical analyses.

#### Immunohistochemistry

For microvessel density analysis, snap-frozen tissues sectioned into  $4\text{-}\mu\text{m}$  specimens were fixed in cold acetone for 10 minutes, blocked with SuperBlock (Scytex Laboratories, UT) for 20 minutes, followed by incubation with a monoclonal rat anti-mouse CD31 against mouse endothelium at a 1:200 dilution (Chemicon, CA) at room temperature for 90 minutes. Sections were sequentially incubated with the secondary antibody, Alexa 594-conjugated goat anti-rat IgG, at a 1:200 dilution (Molecular Probes, OR) at room temperature for 1 hour, and then mounted and examined under fluorescence microscopy. TUNEL assay was performed to assess apoptosis. Paraffin-embedded tumor sections were dewaxed and then subjected to cell permeabilization by



350 W microwave irradiation for 5 minutes. Both basal and Ad vector-induced DNA strand breaks were then labeled with fluorescein-conjugated nucleotides in a terminal deoxynucleotidyl transferase reaction mixture supplemented in the *In situ* Cell Death Detection kit (Roche, IN) at 37 °C for 1 hour, and subjected to reaction with anti-fluorescein-POD conjugate with diaminobenzidine according to the manufacturer's instructions. The samples were counterstained with 1% methyl green to show viable cells.

### Statistical analysis

Differences between treatment groups were analyzed using Student's *t*-test and two-tailed distribution.

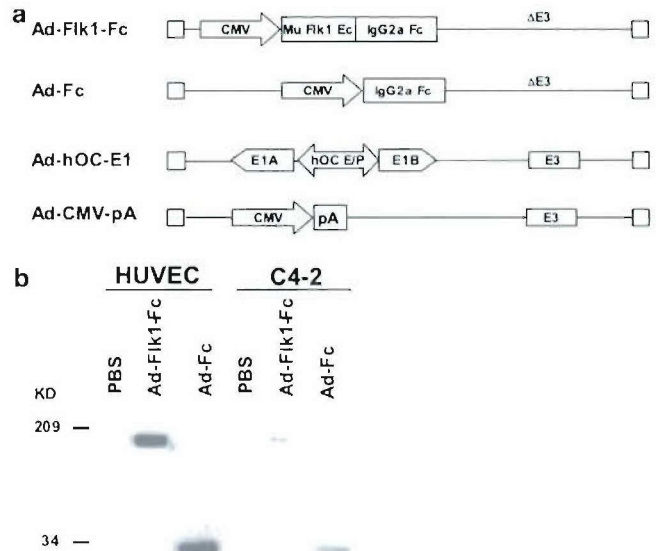
## Results

### Expression of soluble Flk1 on human endothelial and prostate cancer cell lines infected by Ad-Flk1-Fc

To test the antiangiogenic effect of VEGF receptor-based gene therapy in a human prostate cancer model, a replication-defective Ad vector, Ad-Flk1-Fc containing the VEGF receptor-2 (Flk1) cDNA fused with a murine IgG2a Fc fragment (Fig 1a) was used in this study. The gene transduction efficacy of Ad-Flk1-Fc in human prostate cancer cells (C4-2) and endothelial cells (HUVEC) was determined by Western blot of the secreted Flk1 protein in the CM from Ad-Flk1-Fc-infected cells. Both HUVEC and C4-2 cells effectively produced Flk1-Fc fusion protein (185 kDa) or Fc (25 kDa) protein into CM when cells were infected with 10 MOI of Ad-Flk1-Fc or control vector Ad-Fc, respectively (Fig 1b). The quantification of protein expression shown in Western blot demonstrated a six-fold higher susceptibility of HUVEC to Ad infection in comparison with C4-2 cells, which may be due to the higher level of CAR<sup>26</sup> and integrin receptor expressed on the cell surface of HUVEC cells (data not shown).

### Biological effect of soluble Flk1 expression by C4-2 cells on HUVEC cell proliferation, migration and tubular formation *in vitro* following Ad-Flk1-Fc infection

To evaluate the autocrine and paracrine effect of Ad-Flk1-Fc on tumor vasculature, vascular endothelial cells *in vitro*, HUVEC proliferation, migration and tubular formation were assessed when cells were exposed to adenoviruses and/or CM from Ad-infected C4-2 cells. As shown in Figure 2a, direct infection of HUVEC with 10 MOI of Ad-Flk1-Fc inhibited cell proliferation by 25% compared with mock-treated cells. A massively inhibitory effect (65%) on proliferation of HUVEC was observed when cells were incubated with CM from Ad-Flk1-Fc-infected C4-2. No difference was seen among mock-treated, Ad-Fc-infected and Ad-Fc-infected C4-2 CM-treated cells. The paracrine effect of secreted Flk1 by Ad-Flk1-Fc-infected C4-2 cells on the migration of HUVEC was also determined by wound healing assay. The results demonstrated that HUVEC cells incubated with CM from C4-2 cells infected with either vehicle control (PBS) or Ad-Fc moved toward



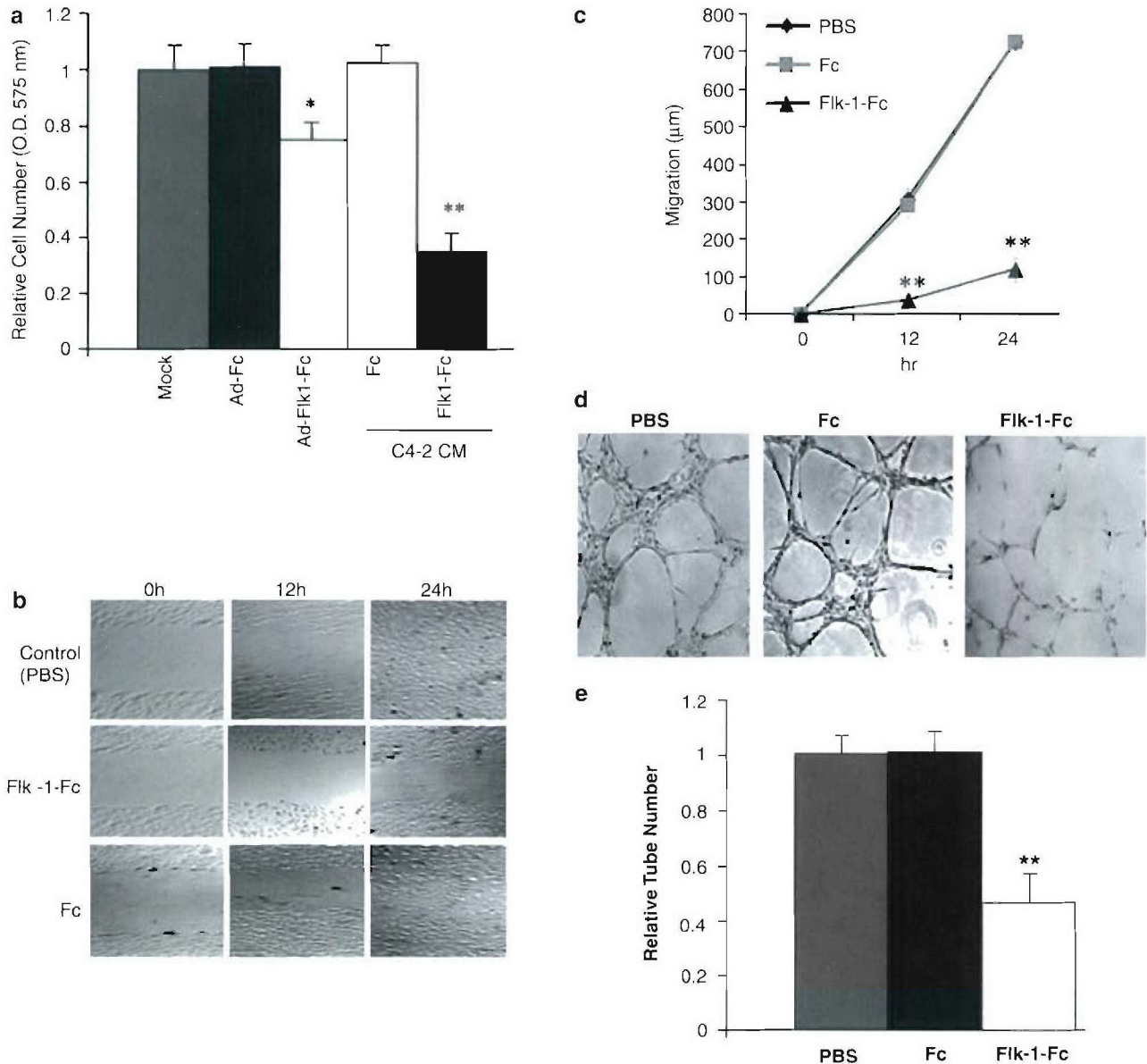
**Figure 1** (a) Structural organization of the Ad vectors used in the present study. An E1- and E3-deleted antiangiogenic Ad5 vector, Ad-Flk1-Fc, bearing a murine Flk1 cDNA sequence encoding both the signal peptide and the ectodomain (Mu Flk1 Ec) at the 5' end fused to a murine IgG2a Fc fragment at the 3' end, and its vector control construct, Ad-Fc, under control by human cytomegalovirus (CMV) promoter and the rabbit  $\beta$ -globin intron and polyadenylation signal (pA). The structure of replication-competent Ad vector Ad-hOC-E1 was described previously.<sup>22</sup> Ad5 E1A and E1B expression are driven by a bidirectional hOC enhancer/promoter (E/P) and terminated by the SV40 pA. A replication-defective Ad-CMV-pA served as a vector control for Ad-hOC-E1. (b) Western blot analysis of Flk1 expression. In all 20  $\mu$ g of concentrated CM from HUVEC and C4-2 cells infected with Ad-Flk1-Fc, control vector Ad-Fc, or mock infection (PBS) was subjected to Western blot using a polyclonal antibody recognizing murine IgG2a Fc as described in Materials and methods. Soluble Flk1-Fc and Fc were detected as distinct bands with the expected apparent MW of 185 and 25 kDa, respectively.

and filled in a  $\sim$ 800- $\mu$ m-wide cell-free zone in a time-dependent manner. Similar effects were also seen in the presence of CM from Ad-Fc-infected C4-2, whereas CM from Ad-Flk1-Fc-infected C4-2 was able to abolish the movement of HUVEC over a 24-hour incubation period (Figs 2b, c). In addition to proliferation and migration assays that describe the initial steps of endothelial cell activation, the ability of HUVEC cells to form tube-like structures on a Matrigel membrane in culture dishes was markedly reduced upon the addition of CM from Ad-Flk1-Fc but not Ad-Fc-infected C4-2 as assessed by morphogenic assay (Fig 2d). Quantitative analysis showed that Ad-Flk1-Fc inhibited tubular formation of HUVEC by 50% in comparison with the controls (Fig 2e).

### Inhibition of prostate cancer cell growth by Ad-Flk1-Fc *in vitro*

Recently, a potential autocrine role for VEGF in prostate cancer was reported.<sup>27</sup> To evaluate whether blocking the





**Figure 2** Inhibition of HUVEC cell growth, migration and tubular formation by Flk1-Fc in an autocrine and/or paracrine manner. **(a)** Inhibition of HUVEC proliferation by Flk1-Fc isolated from HUVEC, infected by Ad-Flk1-Fc but not Ad-Fc at an MOI of 10 in an autocrine manner. A marked inhibition of the growth of HUVEC was observed when exposed to C4-2 CM (10  $\mu$ g/ml for 72 hours), which were harvested from C4-2 cells infected with Ad-Flk1-Fc but not Ad-Fc, in a paracrine manner. Cell proliferation was determined by MTT assay. Data are expressed as means of absorbance at 575 nm and SD of four independent experiments, versus mock-infected group: \* $P < .05$ ; \*\* $P < .005$ . **(b and c)** Inhibition of HUVEC migration by Flk1-Fc. HUVEC migration was determined in a wound-healing assay as described in Materials and methods. **(b)** Representative photomicrographs of HUVEC migration under the influence of vehicle (control-PBS) and Flk1Fc and Fc secreted by C4-2 cells infected with Ad-Flk1-Fc (Flk-Fc) or Ad-Fc (Fc) at the indicated times, 0, 12 and 24 hours as shown ( $\times 25$  magnification). **(c)** Results in panel **b** were confirmed by a quantitative measurement of cell migration. Data are expressed as mean  $\pm$  SD of three independent experiments versus PBS control: \*\* $P < .005$ . **(d and e)** Inhibition of HUVEC tubular formation by infecting HUVEC cells with Ad Flk1-Fc but not Ad-Fc. **(d)** Photomicrograph of HUVEC incubated with control (PBS) on a Matrigel-coated well for 72 hours showed extensive tubular formation ( $\times 40$  magnification). The tubular formation was markedly inhibited by Flk1-Fc and not Fc harvested from C4-2 cells infected by their respected Ad vectors as described above. **(e)** Results of panel **d** were confirmed by a quantitative counting of the number of connecting branches between two discrete endothelial cells. Data represent means  $\pm$  SD of four independent experiments, versus mock-infected group: \*\* $P < .005$ .

autocrine-mediated VEGF pathway could inhibit prostate cancer cell growth, we first confirmed the expression of VEGF (736 pg/ml) in prostate cancer C4-2 cells by ELISA and its type II receptor, Flk1, by RT-PCR (data not

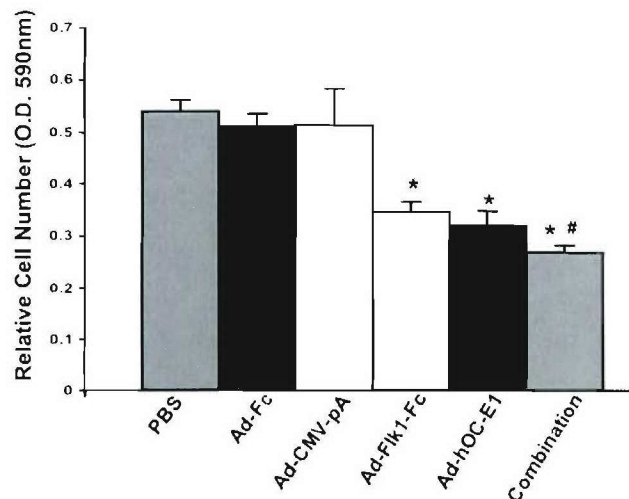
shown). The cell proliferation of C4-2 cells infected with either antiangiogenic Ad-Flk1-Fc, replication-competent Ad-hOC-E1, or both, and control vectors, Ad-Fc and Ad-CMV-pA (construct, see Fig 1a) were determined and



compared with that of mock-infected cells after 72 hours of incubation. As expected, control vectors Ad-Fc and Ad-CMV-pA barely inhibited C4-2 growth but Ad-hOC-E1 effectively replicated and lysed C4-2 cells ( $P < .001$ ) when compared with mock-infected C4-2 cells. Interestingly, Ad-Flk1-Fc also suppressed cell growth significantly ( $P < .001$ ). The treatment of C4-2 with Ad-Flk1-Fc and Ad-hOC-E1 together enhanced further cytotoxicity beyond that of the cells treated with either Ad-Flk1-Fc or Ad-hOC-E1 alone ( $P < .05$ ). These results demonstrated that Ad-Flk1-Fc directly targeted not only angiogenesis but also prostate cancer cell growth. A potential additive effect of Ad-Flk1-Fc oncolytic Ad vector on prostate cancer cell death was therefore examined (Fig 3).

#### "Bystander" antiangiogenic effects of Ad-hOC-E1 in 3D prostate organoid in vitro

Previous studies have demonstrated an increased expression of angiogenesis-stimulating factors such as VEGF, platelet-derived growth factor and transforming growth factor in prostate carcinoma.<sup>28-30</sup> These results suggest that prostate cancer affects endothelial proliferation through the overexpression of these angiogenic growth factors. To determine if the stimulatory effect of prostate cancer cells on endothelial cell proliferation and tubular formation is blocked by the elimination of cancer cells, we established a 3D prostate organoid model that consists of C4-2-GFP, DiI-labeled HUVEC cells or both grown on Matrigel substratum, followed by confocal imaging. As



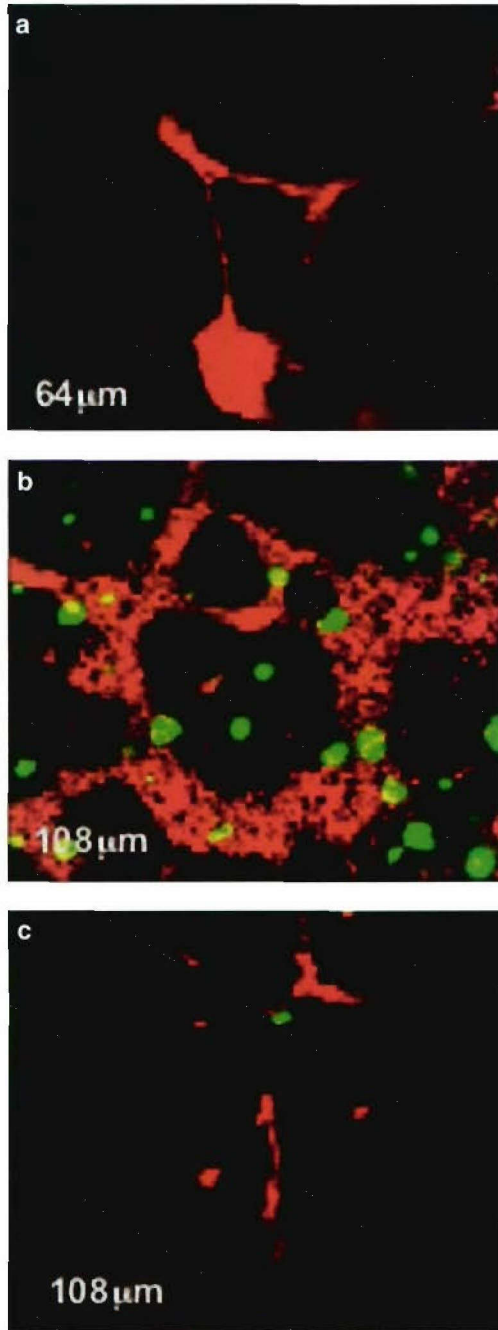
**Figure 3** Cytotoxicity of C4-2 cells resulting from infection with Ad vectors *in vitro*. The relative number of C4-2 cells measured by OD 590nm were tabulated after exposing these cells to PBS (mock infection) or infection by Ad-Flk1-Fc (10 MOI), Ad-Fc (10 MOI), Ad-hOC-E1 (2 MOI) or Ad-CMV-pA (2 MOI) alone or combined with Ad-Flk1-Fc plus Ad-hOC-E1 (combination). The cytotoxicity induced by Ad vectors was assessed by the use of crystal violet staining. Statistical analyses were performed to determine the significance of growth inhibition following therapeutic Ad vector-treatment versus mock-infected group: \* $P < .001$ ; versus Ad-Flk1-Fc or Ad-hOC-E1 group: # $P < .05$ .

expected, HUVEC cultured in 3D Matrigel organized into an irregularly vasculogenic-like network with a total branch extension of  $85 \pm 7 \mu\text{m}$  by confocal microscopy analysis (Fig 4a). The length of network formed by HUVEC increased when HUVEC was cocultured with C4-2-GFP cells ( $450 \pm 71 \mu\text{m}$ ), demonstrating the intrinsic growth of the prostate tumors as well as their inductive role on the proliferation and tubular formation of tumor vasculature (Fig 4b). Preinfection of C4-2-GFP cells with Ad-hOC-E1, which has been demonstrated to replicate selectively in OC-expressing but not non-OC-expressing cells,<sup>22</sup> showed not only a decreased growth of C4-2-GFP but also reduced network formation by HUVEC ( $175 \pm 3.5 \mu\text{m}$ ) in coculture (Fig 4c), whereas no difference was seen in HUVEC prostate organoid with C4-2-GFP cells preinfected with vector (data not shown). Taken together, these results showed prostate cancer-induced vasculogenesis of endothelial cells for the first time, and demonstrated a "bystander" antiangiogenic effect of Ad-hOC-E1 on endothelial cells (non-OC-expressing cells) by the elimination of their supporting prostate cancer cells (OC-expressing cells).

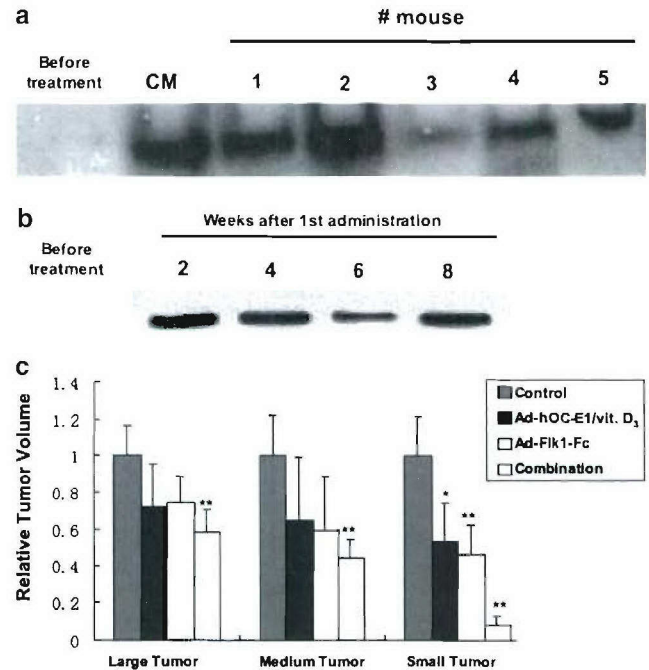
#### Combination of Ad-Flk1-Fc and Ad-hOC-E1 plus vitamin D<sub>3</sub> markedly decreased prostate tumor growth in athymic mice

We evaluated the antitumor effect of Ad-Flk1-Fc and Ad-hOC-E1 plus vitamin D<sub>3</sub> either applied alone or in combination on the growth of subcutaneous C4-2 tumors in athymic mice. To determine whether adenovirus-encoded Flk1-Fc can be efficiently expressed by pre-existing prostate tumors and secreted into blood circulation over an extended period using an intratumoral gene delivery approach, we analyzed plasma samples from tumor-bearing mice that had received intratumoral injections of  $2 \times 10^9$  PFU of Ad-Flk1-Fc twice per week for 2 weeks. Blood samples from individual mice (Fig 5a) or harvested at different time points (Fig 5b) were analyzed. High levels of Flk1-Fc were found in the majority of mouse plasma from Ad-Flk1-Fc-treated animals at day 1 after the last administration and for more than 6 weeks thereafter. These data demonstrated that high levels of Flk1-Fc in the mouse blood circulation were achieved by adenoviral vector-mediated intratumoral gene delivery. To investigate the possible effect of tumor burden on the subsequent therapeutic response of chimeric tumors to Ad vectors, tumor-bearing mice were separated into small, medium and large size groups based on the established tumor volume ( $\sim 50$ ,  $\sim 200$  and  $\sim 500 \text{ mm}^3$ , respectively prior to treatment). C4-2 tumor xenografts were treated with intratumoral Ad-Flk1-Fc and intravenous Ad-hOC-E1 either alone or together. Ad-hOC-E1-treated mice were given i.p. vitamin D<sub>3</sub> to augment the oncolytic activity of Ad-hOC-E1 according to the pre-established protocol.<sup>22</sup> As shown in Figure 5c, both Ad-Flk1-Fc and Ad-hOC-E1 plus vitamin D<sub>3</sub> alone slightly reduced C4-2 tumor growth in the large- and medium-sized tumor groups, but the change was not statistically significant ( $P > .05$ ). In contrast, combination





**Figure 4** Antivascogenic effect on HUVEC growth in the presence of Ad-hOC-E1- and Ad-Flk1-Fc-infected AI prostate cancer cells in a 3D Matrigel coculture system. In this study, HUVEC was tagged by a membrane dye, Dil and C4-2 cells were stably labeled with GFP. A representative photograph shows the growth of HUVEC in Matrigel forming a 3D architecture at Day 3 using a laser scanning confocal microscopy ( $\times 100$  magnification, panel **a**). Coculture of HUVEC and C4-2GFP showed marked vasculogenic stimulation of HUVEC (panel **b**). Under the same coculture conditions, by infecting C4-2GFP cells with Ad-hOC-E1, marked destruction of tubular formation and prostate cancer cell growth was observed (panel **c**). Individual images are labeled and the distance from the bottom of the HUVEC grown is indicated.



**Figure 5** Expression of circulating Flk1-Fc in mice treated with intratumoral injection of Ad-Flk1-Fc and inhibition of pre-established C4-2 tumor growth by the administration of Ad-Flk1-Fc and/or Ad-hOC-E1. **(a)** Level of circulating serum Flk1-Fc, as detected by Western blots in five mice treated with Ad-Flk1-Fc. Tumor-bearing mice (#1 to #5) were treated with Ad-Flk1-Fc intratumorally twice per week for 2 weeks. Plasma levels of Flk1-Fc were measured before treatment and after treatment (at the end of the first week after the second injection of the Ad vector); 20  $\mu$ g of CM from Ad-Flk1-Fc-infected HUVEC cells (CM) served as positive control as described in Materials and methods. **(b)** Persistent expression of Flk1-Fc in mice from week 2 to week 8 after a 2-week Ad-Flk1-Fc treatment of a C4-2 tumor grown in mouse #2. **(c)** Antitumor efficacy of Ad-Flk1-Fc, Ad-hOC-E1, or combination on C4-2 tumor xenografts grown subcutaneously in nude mice. Three groups of mice bearing small, medium and large tumor burdens were treated with Ad-Flk1-Fc or Ad-hOC-E1, either alone or together (combination), with PBS treatment serving as control for an 8-week treatment protocol. The size of the tumor at the termination of the study was measured and presented as mean (percentage of control) tumor volume  $\pm$ SD of each treatment group,  $n=8-10$ . \* $P<.05$  compared with the control-treated tumors.

therapy with Ad-Flk1-Fc and Ad-hOC-E1 plus vitamin D<sub>3</sub> markedly inhibited C4-2 tumor growth, with a 40–60% size reduction in the large- and medium-sized tumor groups ( $P<.005$ ). In the small-sized tumor group, in addition to statistically significant tumor regression observed by Ad-hOC-E1 plus vitamin D<sub>3</sub> ( $P<.05$ ) and Ad-Flk1-Fc or combination of Ad-Flk1-Fc plus Ad-hOC-E1/vitamin D<sub>3</sub> ( $P<.005$ ), three of 10 (30%) of the pre-established tumors completely regressed in athymic mice receiving combination therapy.

#### *Inhibition of angiogenesis and induction of tumor cell apoptosis in vivo*

To determine whether the reduction in tumor growth was associated with a corresponding decrease in vascular

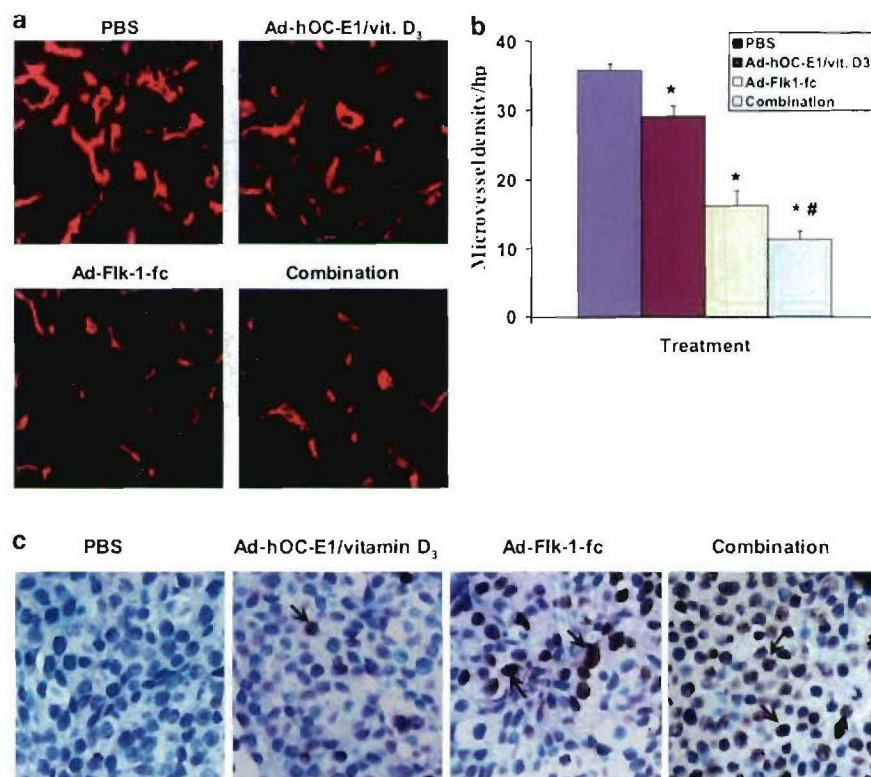


density and enhanced apoptosis, representative tumor specimens receiving different treatments were sectioned for immunohistochemical analysis of blood vessel density (CD31) and apoptosis (TUNEL assay) at the end of treatment. C4-2 tumors from animals receiving PBS showed intense staining for CD31 (Fig 6a), indicating the presence of extensive angiogenesis in the tumor. A significant reduction in CD31-stained vessels was demonstrated after therapy with Ad-hOC-E1 plus vitamin D<sub>3</sub>, but the effect was much more remarkable with Ad-Flk1-Fc, particularly when used in combination with Ad-hOC-E1 (Fig 6a). Quantification of the results reveals a reduction in microvessel density of 20, 50 and 70% in tumors treated by Ad-hOC-E1 plus vitamin D<sub>3</sub>, Ad-Flk1-Fc alone, and combination therapy, respectively, in comparison with the vehicle-control mice (Fig 6b). Furthermore, TUNEL staining of tumor sections from the different treatment groups revealed an increase in tumor specimens (Fig 6c) harvested from hosts treated with Ad-hOC-E1 plus vitamin D<sub>3</sub>. Likewise, tumor specimens harvested from mice treated with either Ad-Flk1-Fc alone or combination therapy showed a progressive increase of cells stained strongly positive by TUNEL (Figs 6b, c). These results suggest that treatment of C4-2 tumors with Ad-hOC-E1 plus vitamin D<sub>3</sub> and Ad-

Flk1-Fc together inhibited the proliferation and tubular formation of tumor-associated vasculature, and also increased apoptosis in both tumor and its vasculature compartments. The regression of implant tumor xenografts and their eventual therapeutic "cure" can be accounted for by the cotargeting effect of this novel therapy on both tumor cells and their associated endothelium.

## Discussion

Inhibiting tumor growth by attacking the tumor vascular supply is a promising concept in cancer gene therapy. However, our results (Fig 5c) and those of others<sup>31</sup> have demonstrated that targeting a single-cell compartment, such as the tumor-associated vascular endothelium, may not be sufficient to generate an effective and durable response. Eventually tumor cells become resistant and refractory to such treatment and augment their anti-apoptotic program.<sup>32</sup> We developed a novel strategy that combines tumor cell-specific targeting to eliminate heterogeneous tumor cell populations, using a conditional replicating Ad-hOC-E1 Ad vector, with tumor endothelium-specific targeting that uses an Ad vector secreting a



**Figure 6** Inhibition of tumor angiogenesis and increase of tumor cell death by treatment with Ad vectors. Tumor-bearing mice in the small tumor group were killed at the end of treatment protocol and tumor tissues were harvested and evaluated. Microvessel density was determined by anti-CD31 staining. (a) Representative photomicrograph showing microvessels in tumors from animals receiving the indicated treatment ( $\times 100$  magnification). (b) Quantitative analysis of microvessel density was made by counting the positive stained cells in 10 high power fields (HPF,  $\times 400$  magnification). Data are given as mean  $\pm$  SD of cell number/HPF. \* $P < .005$  compared with the control group receiving PBS; # $P < .05$  compared with either the Ad-hOC-E1/vit. D<sub>3</sub> or Ad-Flk1-Fc treatment group. (c) Representative photomicrograph of TUNEL staining showing a marked increase in apoptotic cells (see arrows) particularly in Ad-Flk1-Fc and combination treatment groups ( $\times 200$  magnification).



polypeptide, Flk1-Fc, to interfere with the VEGF axis. In the present study, we exploited the obvious advantages of using the conditional replication-competent adenoviruses we developed<sup>8,22</sup> together with an antiangiogenic therapy developed by other investigators<sup>21,33</sup> to treat primary and metastatic prostate cancer. Based on the well-established reciprocal cellular interaction between prostate cancer cells, cancer-associated stroma and tumor endothelium, this cotargeting approach has the potential to inhibit cancer growth, progression and metastasis.<sup>6,34</sup> Results from our studies provide further evidence that cotargeting tumor cells along with the tumor microenvironment is more effective and yields a more durable response than targeting a single-cell compartment.

Compared to other strategies currently under clinical evaluation for the treatment of prostate cancer and its metastasis, such as the use of small molecule inhibitors or monoclonal antibodies in combination with conventional antitumor drugs (reviewed by Retter et al<sup>32</sup>), our approach combined an adenovirus that targets the VEGF axis in the tumor-associated neovascular endothelium with a conditionally replicating oncolytic adenovirus plus vitamin D<sub>3</sub> that targets tumor epithelium. This approach has several advantages. (1) The growth of both prostate cancer and its tumor-associated endothelium is interrupted. (2) The high infection rates with Ad vectors make them ideal candidates for reaching both the tumor and its associated endothelium. (3) The selectivity of the Ad vectors targeting both the tumor and its associated endothelium will cause minimal damage to normal host tissues. We previously demonstrated that OC promoter-mediated oncolytic adenoviruses have a broad spectra of cell-killing activity causing lysis in PSA-producing and nonproducing prostate tumors and renal cell carcinoma, bone, and prostate stromal cells *in vitro*.<sup>8,22</sup> Vitamin D<sub>3</sub> has been shown to inhibit prostate cancer growth as a single agent.<sup>35</sup> When combined with Ad-hOC-E1, vitamin D<sub>3</sub> enhanced adenoviral replication by its inductive role on hOC promoter.<sup>22</sup> Moreover, several experimental studies have demonstrated the direct inhibitory effect of vitamin D<sub>3</sub> on tumor-induced angiogenesis.<sup>36–38</sup> An adenoviral vector bearing a soluble Flk1 receptor has been reported to have the capacity to reduce tumor burden correlated with the degree of MVD in experimental animal models.<sup>21,33,39</sup>

We have taken a mechanism-based approach to demonstrate for the first time the *autocrine* and *paracrine* effect of Ad-Flk1-Fc on tumor and tumor vasculature *in vitro* (Figures 2 and 3). This potent combination caused tumor xenograft regression by “starving” the tumor cells, blocking tumor blood supplies while exerting a direct cell-killing effect on the tumor cells themselves (Figures 5 and 6).

This finding is consistent with a previous report<sup>27</sup> where LNCaP cells stimulated with recombinant VEGF induced DNA synthesis and recruited quiescent cells into the S-phase of the cell cycle via signaling through cell surface Flk1 receptors. Moreover, as hypothesized by other investigators,<sup>40,41</sup> the inability of replication-defective Ad-Flk1-Fc to diffuse through tissues may be complemented by acquiring the ability to multiply via the

exogenous adenoviral E1 gene product provided by Ad-hOC-E1 within tumor cells when they are cotargeted with both of these Ad vectors. Combining Ad-hOC-E1, vitamin D<sub>3</sub> and Ad-Flk1-Fc therefore represents an effective therapeutic approach to prostate cancer.

To succeed, a gene therapy-based approach to the treatment of cancer must be not only effective, but also safe and practical. Unlike small molecule inhibitors or anti-VEGF monoclonal antibodies, which require prolonged administration to achieve a long-term steady-state therapeutic level of the protein, a single intravenous injection of Ad vector expressing a secretable antiangiogenic protein is sufficient to achieve a persistent level of gene products in mouse serum.<sup>21,33,42</sup> However, the downside of this strategy is that systemic gene delivery by an Ad vector could cause hepatotoxicity from the overexpression of antiangiogenic factors in hepatocytes.<sup>43</sup> By contrast, in our study the local intratumoral injection of Ad-Flk1-Fc achieved both local and systemic accumulations of Flk1-Fc for at least 6 weeks with adequate suppression of neovascularization in C4-2 tumors via autocrine and/or paracrine blockade, and with no evidence of organ toxicity. Consistent with the observation by other groups<sup>43</sup> that i.p.-delivered adenovirus-mediated soluble FLT-1 caused no significant systemic cytotoxicity, our experiments support the use of Ad vectors delivering Flk1-Fc as local therapy for regional *in vivo* antiangiogenic prostate cancer gene therapy.

Toxicity studies of the systemic delivery of Ad-hOC-E1 have been hampered historically by the lack of an adequate experimental animal model, because human adenoviruses replicate only in human cells. However, OC promoter activity has largely been restricted to osseous tissues with marginally detectable levels in the brain of transgenic animals, using 3.9 kb of human OC promoter construct fused to the CAT reporter.<sup>44,45</sup> The safety of an adenoviral vector carrying OC promoter-driven HSV-TK gene was also recently demonstrated in a Phase I clinical trial.<sup>46</sup>

The major side effect of high-dose vitamin D administration is hypercalcemia, which would jeopardize its clinical utility. Although the vitamin D<sub>3</sub> analogue Ro 25-9022, used in the present study, has not been tested at the maximum tolerated dose, when mice treated with 4 ng of Ro 25-9022 twice per week were compared with untreated mice, they showed only a mild side effect, a less than 10% body weight reduction for a period of 3 weeks. The mice recovered after the vitamin D<sub>3</sub> treatment was stopped, which is consistent with our previous study.<sup>22</sup> This minor side effect could be further overcome by using other structural analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub> that have been developed, which show significant antiproliferative activity but a less calcemic effect *in vivo*.<sup>47</sup>

The results of our work suggest that combination therapy with conditional replication-competent adenovirus and an antiangiogenic gene therapy targeting the VEGF axis may have clinical utility for prostate cancer treatment. Several issues remain to be addressed, some of which cannot be examined using the currently available animal models. In practice, intratumoral administration



of the adenovirus in cancer patients may result in the production of neutralizing antibodies with subsequent elimination of infected cells, particularly after multiple treatments. However, additional virus-induced cytotoxic effects might prove beneficial in some instances. It has been shown that the recruitment and stimulation of tumor-specific T lymphocytes can lead, in some cases, to systemic antitumor immunity.

Experimental data from several other laboratories provided intriguing evidence that aggressive breast and prostatic carcinoma and melanoma tumors demonstrate vasculogenic mimicry,<sup>48-50</sup> by which tumor cells form *de novo* vasculogenic-like networks *in vitro* in the absence of endothelial cells or fibroblasts, concomitant with the expression of several vascular-associated markers including thrombin receptor, endothelin-B receptor, endoglin, TIE-2 or Flk1.<sup>51</sup> In addition to *in vitro* cell models, tumor cell-lined vasculature is detectable in clinical specimens,<sup>52,53</sup> suggesting an important role for vasculogenic mimicry in the establishment, growth and metastasis of aggressive human tumors. We have observed that the ability of prostate cancer cell lines to form patterned vasculogenic-like networks in 3D Matrigel culture *in vitro* is associated with their tumorigenicity *in vivo* (Jin, unpublished data). Additional molecular studies are in progress in our laboratory to elucidate the molecular mechanism responsible for vasculogenic mimicry. Based on the expression of Flk1, one of the key vascular markers in C4-2 cells, it is tempting to speculate that the induction of tumor cell apoptosis by Ad-Flk1-Fc shown in our study may be partly attributable to blocking the formation of *tumor-lined vasculature* in addition to endothelial-lined vasculature.

In summary, we have demonstrated that the adenovirus-mediated intratumoral transfer of an ectodomain of the Flk1 gene is an efficient means of delivering gene therapy to both vascular endothelial cells and prostate cancer cells, resulting in suppressed tumor growth via an indirect antiangiogenic mechanism and/or direct tumor cell kill. Our results showed that eliminating cancer cells with a conditional replication-competent adenovirus could improve the therapeutic effectiveness of Ad-Flk1-Fc through Ad-vector-induced oncolysis and subsequent Ad infection of tumor cells and their adjacent neovasculature. Our "proof-of-concept" study establishes for the first time that the adenovirus-mediated cotargeting of prostate cancer and tumor endothelium may be an effective strategy for destroying androgen-independent and metastatic human prostate tumors. Future studies are planned to improve the therapeutic effects of combination therapies for the eradication of larger sizes of prostate tumors.

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